PHYSIOLOGICAL CONTROL OF *DROSOPHILA* OVARIAN STEM CELL LINEAGES

By
Kaitlin M. Laws

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ABSTRACT

In order to support tissue function, adult stem cell activity must respond to organismal dietary status and whole-body physiology. The complex signaling networks impinging on stem cells, however, are not fully understood. The focus of this dissertation is how *Drosophila* ovarian stem cell lineages, which have well-characterized responses to diet, sense and respond to their physiological environment. In mammals, adipocytes have a key endocrine role, mediated in large part through secreted peptide hormones called adipokines. I describe an intrinsic requirement for the *Drosophila* homolog of a mammalian adipokine receptor, the adiponectin receptor (AdipoR), in germline stem cell (GSC) maintenance and demonstrate that overexpression of AdipoR in the germline mitigates age-associated GSC loss. I also explore the distinct, but overlapping, roles of the cellular energy sensor AMP-activated protein kinase (AMPK) in ovarian stem cell lineages. In addition to nutrient-dependent roles in cell proliferation and growth in these cells, AMPK intrinsically controls the function of cells in the somatic lineage by a diet-independent mechanism. In complementary studies, I address the regulatory role of the fat body in oogenesis. With Dr. Alissa Armstrong, a postdoctoral fellow in the lab, I characterize two mechanisms of amino acid sensing in adult adipocytes that affect distinct stages of oogenesis. Furthermore, I summarize my efforts toward identifying additional roles for the fat body in oogenesis, including AdipoR signaling and lipid storage in that tissue. I outline possible methods for the identification of the *Drosophila* adiponectin-like ligand. Finally, I provide a proof of principle for a screen to identify novel diet-dependent factors regulating *Drosophila* oogenesis using a laboratory strain of yeast. This dissertation sheds light on the dense physiological signaling networks regulating stem cells and their progeny and represents substantial progress toward understanding inter-organ communication.

Advisor: Daniela Drummond-Barbosa, Ph.D.

Readers: Philip Jordan, Ph.D.
Erika Matunis, Ph.D.
Alan Scott, Ph.D.

Alternates: William Wright, Ph.D.
Gary Ketner, Ph.D.
G. William Wong, Ph.D.

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β-gal:	β galactosidase (LacZ)
20E :	20-hydroxyecdysone
4E-BP:	Eukaryotic translation initiation factor eIF4e binding protein
A. aegypti:	*Aedes aegypti*
A2BP1:	Ataxin-2 binding protein 1
AAR:	Amino acid response
Aats:	Aminoacyl tRNA synthetase
AdipoR:	Adiponectin receptor
ADP:	Adenosine diphosphate
Adp:	Adipose
AKT/PKB:	Protein kinase B
AMP:	5' adenosine monosphosphate
AMPK:	AMP-activated protein kinase
ANOVA:	Analysis of variance
APC:	Anaphase promoting complex
AR:	Androgen receptor
ATF4:	Activating transcription factor 4
Atg:	Autophagy related gene
ATP:	Adenosine triphosphate
arm-LacZ:	Armadillo LacZ
BAC:	Bacterial artificial chromosome
Bam:	Bag of marbles
BMP:	Bone morphogenic protein
BrdU:	5-bromo-2-deoxyuridine
BSA:	Bovine serum albumin
C. elegans:	*Caenorhabditis elegans*
CAMKKβ:	Calmodulin-dependent protein kinase kinase β
CC3:	Cleaved caspase 3
cDNA:	Complementary deoxyribonucleic acid
Ced 1/2:	Cell death abnormal 1/2 (*C. elegans*)
CySC:	cyst stem cell
DAPI:	4',6-diamidino-2-phenylindole
Dcp-1:	Death caspase-1
DGRC:	*Drosophila* Genomics Resource Center
Df:	Deficiency
DIAP1:	*Drosophila* inhibitor of apoptosis protein 1
DILP:	*Drosophila* insulin-like peptide
DNA:	Deoxyribonucleic acid
Dpp:	Decapentaplegic
DSHB:	Developmental Studies Hybridoma Bank
DTC:	Distal tip cell

Dronc*:*	*Drosophila* Nedd2-like caspase
E74:	Ecdysone-induced protein 74EF
E75:	Ecdysone-induced protein 75B
E78:	Ecdysone-induced protein 78C
E-cad:	E-cadherin
EcR:	ecdysone receptor
EdU:	5-ethynyl-2-deoxyuridine
ERK:	Extracellular signal-regulated kinase
FB:	fat body
FLP:	flippase
FOXO:	forkhead box, sub-group "O"
FRT:	flipase recognition target
FSH:	follicle stimulating hormone
FSC:	follicle stem cell
GATOR1:	GAP activity towards rags 1
GCN2:	General control nonderepressible 2
GDNF:	Glial cell-dervied neurotro
GFP:	Green fluorescent protein
GLI1:	Glioblastoma-associated oncogene 1
GnRH:	Gonadotropin releasing hormone
GSC:	GSC
Hh:	Hedgehog
HNF4:	Hepatic nuclear factor 4
Hts:	Hu-li tai shao
IGF:	Insulin-like growth factor
IGFR:	Insulin-like growth factor receptor
ILP:	Insulin-like peptide
Imp-L2:	Ecdysone-inducible gene L2
InR:	Insulin receptor
IRS:	Insulin receptor substrate
LamC:	Lamin C
LH:	Luteinizing hormone
LKB1:	Liver kinase B1
Lsd2:	Lipid storage droplet 2
Lsp:	Larval serum protein
MAPK:	Mitogen-activated protein kinase
MNC:	Medial neurosecretory cell
mRNA:	Messenger ribonucleic acid
MSP:	Major sperm protein
nos:	Nanos
neo^r:	Neomycin resistance
Nprl2/3:	Nitrogen permease regulator-like 2 and 3
Orb:	OO18-RNA binding protein
pAMPK:	Phosphorylated AMP-activated protein kinse
PBS:	Phosphate buffered saline
PBST:	Phosphate buffered saline with Triton X-100

PCR:	Polymerase chain reaction
PGC:	Primordial germ cell
PI3K:	Phosphoinositide-3 kinase
Plzf:	Promyeolocytic leukemia zinc finger
pMad:	Phosphorylated mothers against decapentaplegic
PPARγ:	Peroxisome proliferator-activated receptor γ
PTEN:	Phosphatase and tensin homolog
PTM:	Peritubular myeoid cells
RA:	Retinoic acid
RAR:	Retinoic acid receptor
RHEB:	Ras homolog enriched in brain
RNA:	Ribonucleic acid
RNAi:	RNA interference
Rp49:	Ribosomal protein 49 (standard control from RT-PCR)
RXR:	Retinoid X receptor
Rsks-1:	Ribosomal protein S6 kinase (*C. elegans*)
RT-PCR:	Reverse transcriptase PCR
S. cerevisiae:	*Saccharomyces cerevisiae*
S6K:	Ribosomal protein S6-p70-protein kinase
Slif:	Slimfast
SOD:	Superoxide dismutase
SSC:	Spermatogonial stem cell
TFGβ:	Transforming growth factor β
TOR:	Target of rapamycin
TORC:	Target of rapamycin complex
TSC1/2:	Tuberous sclerosis protein 1 and 2 complex
tRNA:	Transfer ribonucleic acid
UAS:	Upstream activating sequence
Upd:	Unpaired
USP:	Ultraspiracle
VDRC:	Vienna *Drosophila* RNAi Center
W:	White
Y:	Yellow
YPD:	Yeast peptone dextrose

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Organisms face frequent challenges to their homeostasis, and sensing and responding appropriately to these challenges is essential for their survival and successful reproduction. Diet and various stressors in the external environment help determine the levels of many circulating factors, including nutrients, metabolites and hormones, which in turn can influence the germ line, a special lineage that gives rise to gametes and allows species propagation (Ables et al. 2012, Hubbard 2011). Constant evolutionary pressure on reproduction has therefore led to very tight coupling of nutrient availability, metabolic status and other aspects of whole-body physiology to the biology of germ cells.

In many systems, germline stem cells (GSCs) support gametogenesis throughout most of adult life. Germ cell development from the stem cell stage to fully differentiated gametes is energetically costly and entails a large number of cellular processes that impose varying metabolic demands. It is not surprising, therefore, that multiple steps of gametogenesis are regulated by diet and other physiological factors (Ables et al. 2012, Hubbard 2011, Gracida and Eckmann 2013, Busada and Geyer 2016).

Over the past 15 years, many studies have tackled the complex question of how whole-body physiology controls adult GSC lineages by taking advantage of *in vivo* model systems amenable to genetic manipulation. In this Chapter, I summarize and discuss the progress in this field, with a special focus on diet-dependent mechanisms that modulate adult GSC lineages in *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammals. I include an overview of the response of these GSC lineages to diet by intrinsic nutrient-dependent

pathways, diet-dependent hormones, and discuss the regulation of GSC lineages by other organs.

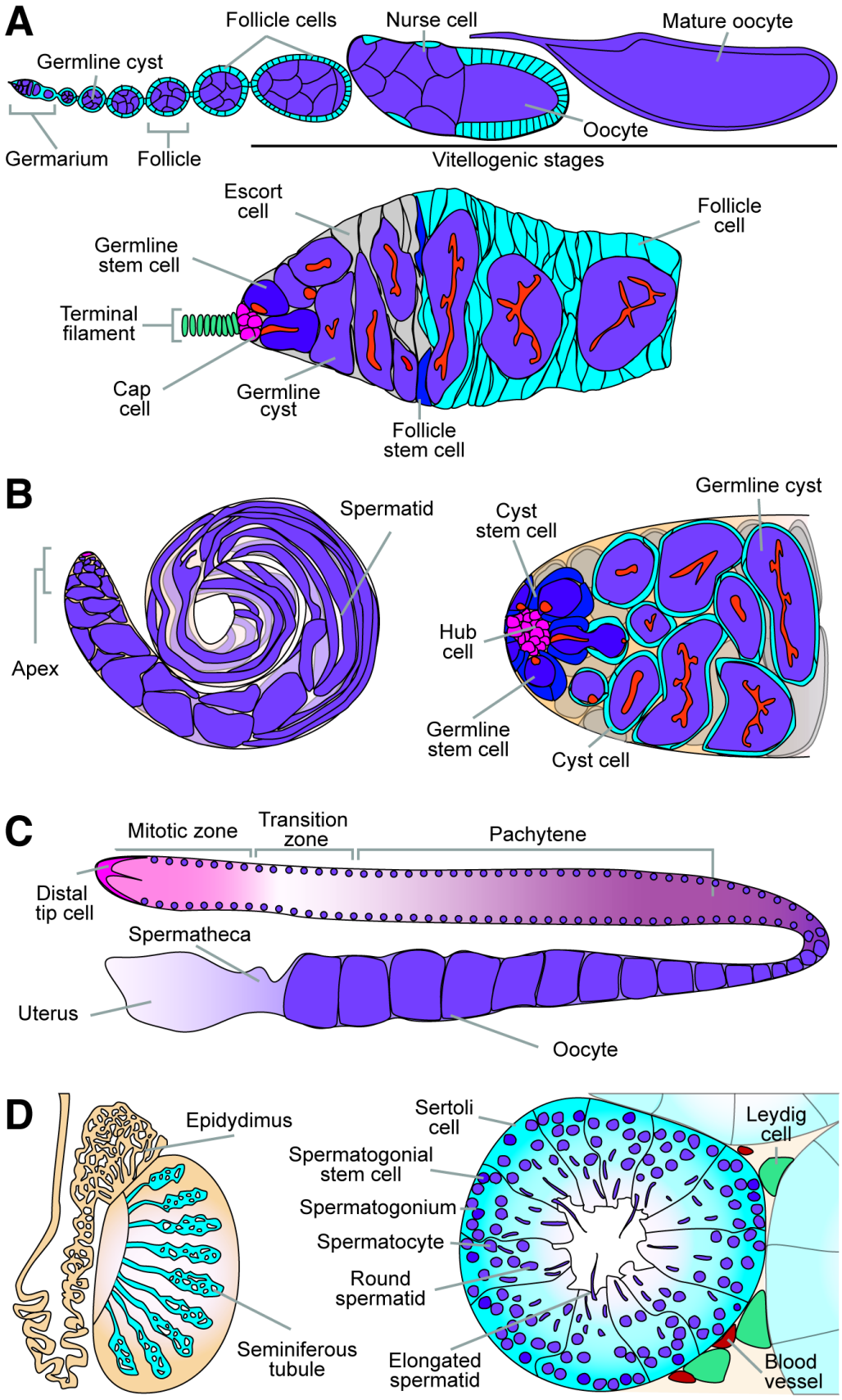
Responses of germline stem cell lineages to diet

By coupling reproduction to nutrient availability, organisms avoid the costly metabolic investment of reproduction under suboptimal conditions and improve evolutionary fitness. Diet-dependent regulation of germ cells is therefore found in a wide range of systems regardless of life history strategy or germline organization. For example, abalone produce fewer gametes under nutrient stress (Rogers-Bennett 2010), starved zebrafish slow down egg production (Wang et al. 2006), and women with anorexia or excessively low body fat do not ovulate (Group 2006, Rojas et al. 2015). In cases where germline stem cells (GSCs) support gametogenesis, the germline can be influenced by diet-dependent signaling in GSCs or their progeny, in the niche (a specialized microenvironment that maintains stem cells), or in intermediate organs that provide relay signals.

The *Drosophila melanogaster* ovary

The *Drosophila* ovary has a well-described cell biology (Spradling 1993). Each ovary contains 15 to 20 ovarioles, composed of progressively more developed egg chambers (or follicles) formed in an anterior germarium, which houses GSCs and follicle stem cells (FSCs) (Figure 1.1A). Two to three GSCs are closely associated with a group of somatic cap cells, which are the major cell type in the GSC niche. Cap cells produce the growth factor bone morphogenetic protein (BMP) that signals to maintain the GSC fate by repressing a differentiation factor, while the physical association between cap cells and GSCs requires E-

Figure 1.1. GSC lineages. (A) Diagram of a *Drosophila* ovariole (top), which contains growing follicles. Each follicle is composed of a germline cyst surrounded by follicle cells and is produced from stem cell populations in the germarium (bottom). Germline stem cells (GSCs; dark purple) are juxtaposed to a somatic niche consisting primarily of cap cells (pink) and terminal filament cells (teal). GSCs divide asymmetrically, and their progeny generate 16-cell germline cysts (light purple) containing one oocyte and 15 nurse cells. The fusome (orange) becomes progressively more branched as cysts divide. Germline cysts initiately associate with escort cells (gray), and are subsequently enveloped by follicle cells (light blue) generated by follicle stem cells (dark blue) to form folicles. **(B)** The *Drosophila* testis (left) is a blind-end tube. GSCs (dark purple) reside at its apical end in close association with hub cells (pink) and cyst stem cells (CySCs, dark blue) (right). GSCs and CySCs divide asymmetrically, and their progeny (germline cysts and cyst cells, respectively) remain associated with each other during spermatogenesis. **(C)** Diagram showing one of the two gonad arms of adult *C. elegans* hermaphrodites. A niche comprising the distal tip cell (DTC; pink) maintains progenitor cells in the mitotic, proliferative zone. As progenitor cells move away from the niche, they enter meiosis. Sperm produced during larval stages are stored in the spermatheca; oocytes (purple) generate later are fertilized by stored sperm (or sperm introduced by mating) before progressing to the uterus. **(D)** In the mouse testis (left), spermatogenesis takes place in seminiferous tubules. Cross-section of a seminiferous tubule (right) showing different stages of the lineage supported by basally located spermatogonial stem cells (SSCs, dark purple). SSCs divide to produce mitotically active differentiating progeny (spermatogonia), which undergo meiosis (spermatocytes) and spermiogenesis (spermatids), and are released into the lumen of the tubule. Sperm undergo further maturation in the epidydimus, where they are eventually stored. Leydig cells (teal), blood vessels (red), and Sertoli cells (blue) plays important roles in support the SSC lineage.


A
Follicle cells
Nurse cell
Mature oocyte
Germline cyst
Oocyte
Germarium
Follicle
Vitellogenic stages
Escort cell
Follicle cell
Germline stem cell
Terminal filament
Cap cell
Germline cyst
Follicle stem cell
B
Spermatid
Apex
Germline cyst
Cyst stem cell
Hub cell
Germline stem cell
Cyst cell
C
Mitotic zone
Transition zone
Pachytene
Distal tip cell
Spermatheca
Uterus
Oocyte
D
Epididymus
Seminiferous tubule
Sertoli cell
Leydig cell
Spermatogonial stem cell
Spermatogonium
Spermatocyte
Round spermatid
Elongated spermatid
Blood vessel

cadherin. Anterior to cap cells, a row of terminal filament cells also contributes to the niche. GSCs typically divide asymmetrically to self-renew and generate daughter cystoblasts. Cystoblasts divide four additional times with incomplete cytokinesis to form a 16-cell cyst: one of these cyst cells acquires an oocyte fate; the others support oocyte development as nurse cells. GSCs and their early progeny are easily identifiable based on the morphology of a specialized structure, the fusome. In GSCs, the fusome contacts the cap cell interface and remains round most of the time; as the cystoblast divides to form 16-cell cysts, the fusome becomes progressively more branched (Xie 2008). Early germ cells are closely associated with escort cells (also known as inner germarial sheath cells), which are required for the proper formation of 16-cell cysts (Kirilly et al. 2011). Two FSCs (abutting the posterior-most escort cells) give rise to follicle cells that envelop each 16-cell cyst to give rise to a follicle that buds off the germarium and proceeds through fourteen developmental stages (Xie 2008).

Drosophila oogenesis is energetically demanding and highly regulated by diet (Ables et al. 2012). On a yeast-rich diet, each female lays an average of 80 eggs per day, but upon shifting to a yeast-free (poor) diet, egg laying rates drop to just one or two eggs daily (Drummond-Barbosa and Spradling 2001). This largely reversible response to diet occurs within 18 to 24 hours, and reflects the concerted regulation of multiple processes in oogenesis. The proliferation rates of GSCs and FSCs, and the proliferation and growth of their progeny decrease, and follicles develop two- to three-fold more slowly on a poor diet (Drummond-Barbosa and Spradling 2001). An additional effect of starvation in developing follicles is the accumulation of large aggregates of processing bodies and cortically enriched microtubules; this is a reversible response that requires microtubule motor proteins (Burn et al. 2015). GSC and cap cell loss over time is also accelerated by a poor diet (Drummond-

Barbosa and Spradling 2001, Hsu and Drummond-Barbosa 2009). In addition, early germline cysts die at an increased frequency within the germarium, follicles entering vitellogenesis degenerate, and ovulation is largely blocked, causing an accumulation of mature stage 14 egg chambers within the ovary (Drummond-Barbosa and Spradling 2001).

The *Drosophila* testis

Drosophila male GSCs reside at the testis apex in a niche, the hub, composed of 10-15 somatic cells (Figure 1.1B). Six to nine GSCs are closely associated with approximately twice as many somatic cyst stem cells (CySCs) at the hub. The cytokine Unpaired (Upd) produced by the hub is required for adhesion of GSCs to the hub, and for maintaining the CySC fate. Additional signals contribute to GSC and CySC maintenance, including BMPs and Hedgehog, respectively (Greenspan et al. 2015). GSCs divide asymmetrically to generate gonialblasts that form two-, four-, eight- and 16-cell cysts, collectively referred to as spermatogonia. CySCs give rise to postmitotic cyst cells; a pair of cyst cells envelops the gonialblast and remains associated with the resulting germline cyst as it develops. Meiotic divisions produce a cyst with 64 spermatids, followed by their individualization and mature sperm generation (Fuller 1993).

Despite the obvious size and energy storage differences between oocytes and sperm, early stages of *Drosophila* spermatogenesis are also regulated by diet. Upon protein starvation, the number of GSCs and their division rates decrease, and these effects are reversible (McLeod et al. 2010). A reduction in protein and sugar levels also slows down GSC proliferation, at least in part as a result of increased rates of centrosome misorientation (Roth et al. 2012). A reduction in overall food intake without specific removal of nutrients has been reported to result in a slight delay in GSC loss with age (Mair et al. 2010),

suggesting that specific dietary manipulations can impact GSC maintenance in unique ways. More recent studies examining the kinetics of the protein-starvation response show that although GSC proliferation and numbers decrease initially, after about a week, GSC numbers stabilize and proliferation rates return to normal levels. During this response, early spermatogonial cells die at the two- to four-cell stage, and their death requires caspase activity in surrounding cyst cells. When death of cysts cells is blocked by *dronc* caspase knockdown or DIAP1 overexpression, GSCs are no longer maintained during prolonged starvation, suggesting that elimination of cyst cells/spermatogonial units has a protective effect against protein starvation (Yang and Yamashita 2015).

The *Caenorhabditis elegans* gonad

The hermaphrodite *C. elegans* has a gonad with two arms each containing ~1000 germ cells and capped by a single somatic distal tip cell (DTC) at each end (Figure 1.1C). Notch ligands produced by the DTC maintain a population of ~225 proliferating germ cells, including a stem cell pool of ~35-70 GSCs, in the distal mitotic zone (Kimble and Seidel 2008). As germ cells move proximately, they enter meiosis as a result of reduced Notch signaling and complex posttranscriptional regulation. Following intense oocyte growth, ovulation occurs, followed by fertilization (by sperm produced during larval development or introduced through mating) and egg laying (Hubbard and Greenstein 2005, L'Hernault 2009) (Figure 1.1C).

Complete removal of food in adult hermaphrodites can lead to different phenotypes (Angelo and Van Gilst 2009). Maternal death may occur due to internal development of progeny (who will eat their way out of the mother) following reduced rates of egg laying. Animals that escape matricide undergo instead an adult reproductive diapause, where over

the initial 10 days of starvation, the germ line is reduced to ~35 germ cells (presumably GSCs) that resist continued starvation for over 30 days and are able to reconstitute a fully functional germ line within 72 hours of refeeding (Angelo and Van Gilst 2009). A more recent study showed that germ cells in the proliferative zone stop dividing and arrest in the G2 phase of the cell cycle, and meiotic entry is inhibited within a few hours of starvation of early adults, and these effects are reversible upon refeeding. Interestingly, GSCs retain their stemness independently of Notch signaling during this starvation-induced arrest (Seidel and Kimble 2015).

The mouse testis

Mouse spermatogenesis takes place in the epithelial lining of seminiferous tubules in the testis (Figure 1.1D) (de Rooij and Russell 2000). Large somatic Sertoli cells span the entire epithelium thereby contacting all stages of germ cell development, and they secrete glial cell line-derived neurotrophic factor (GDNF), required for GSC self-renewal (Franca et al. 2016). GSCs, called spermatogonial stem cells (SSCs), represent a subset of undifferentiated A_{single} spermatogonia located basally in the epithelium. SSCs give rise to daughters that undergo four rounds of incomplete divisions remaining in clusters of two cells (A_{paired}), and four, eight, 16, and up to 32 cells (collectively called $A_{aligned}$ spermatogonia), subsequently differentiating into B spermatogonia (de Rooij and Russell 2000). B spermatogonia differentiate into spermatocytes, which undergo meiosis to form haploid spermatids that differentiate into sperm released into the lumen. A highly vascularized interstitium surrounding seminiferous tubules contains peritubular myoid cells, macrophages, and testosterone-producing Leydig cells, all of which support spermatogenesis (Oatley and Brinster 2012, DeFalco et al. 2015).

Although specific effects on SSCs have not been carefully analyzed, several studies point to possible connections with diet. For example, diet-induced obesity and vitamin D or zinc deficiency can lead to a decrease in male fertility in mice (Fan et al. 2015, Sun et al. 2015, Croxford et al. 2011). Adult mice deprived of dietary vitamin A show testicular degeneration as a result of increased apoptosis and sloughing of immature germ cells into the lumen in more severe cases (Boucheron-Houston et al. 2013). Several of these observations have parallels in humans: obese men are more likely to be infertile (Campbell et al. 2015), and there is a positive correlation between vitamin D and zinc and sperm quality in adult men (Blomberg Jensen 2014, Colagar et al. 2009).

Nutritional control of GSC lineages

GSC lineages sense and respond to nutritional inputs through multiple mechanisms integrated into a seamless physiological output. GSC lineages can directly receive dietary information through cellular energy sensors, or nutrient transport and sensing. These same inputs can act in the niche, indirectly influencing GSCs and their progeny. Finally, remote endocrine cells can produce hormones in a diet-dependent manner with broad physiological influence over the organism, including direct effects on the niche, GSCs, or their differentiating progeny, or indirect effects through one or more intermediate organs.

AMPK and TOR: nutrient sensors that control GSC lineages

Highly conserved nutrient-sensing pathways operate in a wide range of cells, including those in GSC lineages; yet, several examples illustrate how these pathways can control distinct processes depending on the cellular context. The energy sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a heterotrimeric protein, composed of a catalytic α and regulatory β and γ subunits. When ATP levels are low (for

example, due to low nutrients or prolonged exercise), AMP and ADP binding to the γ subunit lead to activation of AMPK, which also requires phosphorylation of the α subunit by liver kinase B1 (LKB1) or, in a few cases, by calmodulin-dependent protein kinase kinase β (CAMKKβ) (Hardie et al. 2016) (Figure 1.2). Phosphatases, including protein phosphatase V in *Drosophila* and yeast (Bozaquel-Morais et al. 2010, Ruiz et al. 2011, Yin et al. 2014) and members of the Manganese-dependent protein phosphatase family have been shown to dephosphorylate AMPK *in vitro* (Voss et al. 2011), but many of the regulatory phosphatases of AMPK are likely unknown. AMP or ADP binding to AMPKγ induces a conformational change that restricts α subunit dephosphorylation, thus promoting AMPK activity (Riek et al. 2008, Sanders et al. 2007). Since different AMPKγ subunits have disparate AMP binding affinities (Cheung et al. 2000), the AMPKγ isoform present in a given AMPK heterotrimer is a major determinant of its activity. In many organisms, multiple genes encode each AMPK subunit; in *Drosophila*, a single gene encodes each subunit (Pan 2002), but multiple splice variants, including 16 currently annotated for *AMPKγ* (www.flybase.org) may similarly confer differences in activity and tissue specificity. For example, the *löchrig* mutation, which leads to dramatic neurodegeneration phenotypes in adult flies, is caused by the disruption of a single, neuron-specific splice variant of *AMPKγ* (Tschape 2002). Canonically, AMPK activation inhibits anabolism and cell growth and promotes catabolic processes to restore cellular energetic balance (Hardie 2015, Hardie and Ashford 2014). Consistent with this role, AMPK in the *Drosophila* larval fat body restricts cell size and promotes developmental autophagy (Voss et al. 2011, Lippai et al. 2008). However, during *Drosophila* embryogenesis, AMPK is globally required for normal cell mitoses and polarity of the epithelium (Lee et al. 2007), and several *AMPK* mutant flies have progressive

neurodegeneration phenotypes (Tschape 2002, Spasic et al. 2008), suggesting the possibility of energy-independent AMPK functions. Indeed, energy-independent AMPK activation has been reported in response to reactive oxygen species (Emerling et al. 2009), and CAMKKβ can promote AMPK activation in the absence of elevated AMP (Woods et al. 2005, Hurley et al. 2005, Hawley et al. 2005), indicating that specific cellular conditions might promote AMPK activity through noncanonical mechanisms.

AMPK inhibits growth in part through the inhibition of the kinase Target of Rapamycin (TOR, or mTOR in mammals). TOR exists as part of two distinct complexes, TORC1 and TORC2, which differ in their regulation and downstream roles (Bar-Peled and Sabatini 2014, Huang and Fingar 2014, Devreotes and Horwitz 2015). TORC1 is the best-understood complex and integrates diverse upstream inputs, including extracellular signals (e.g. insulin signaling) and intracellular cues (e.g. amino acid levels, AMPK activity), to control a variety of downstream cellular processes, including cell growth (Hindupur et al. 2015) (Figure 1.2). AMPK regulates TOR signaling through direct phosphorylation of TSC (Inoki et al. 2002) and, in some cells, phosphorylation of TORC1 (Koo et al. 2005).

TOR signaling is required in *Drosophila* female GSCs. TOR activity promotes GSC proliferation via G2 independently of insulin signaling (see below). Intriguingly, maintenance of female *Drosophila* GSCs requires very precise regulation of TOR signaling levels. *Tor* mutation decreases GSC numbers (LaFever et al. 2010), and loss of *Tsc1* function causes a significantly more severe GSC loss phenotype (LaFever et al. 2010, Sun et al. 2010), indicating the either low or high TOR activity levels are detrimental to stem cell maintenance. *Tsc1* GSCs have low levels of BMP signaling, suggesting an impaired ability to respond to niche signals (Sun et al. 2010). Regulation of the *C. elegans* germline progenitor

pool by AMPK and TOR is mechanistically distinct in developing and adult worms. During earlier nutrient-dependent developmental checkpoints, the homologs of mammalian AMPK, *aak-1* and *aak-2*, suppress germline proliferation, and their simultaneous mutation leads to germline hyperplasia (Fukuyama et al. 2012, Narbonne and Roy 2006). The *C. elegans* homolog of the TOR substrate (S6K), *rsks-1,* is required for germ cell proliferation during the fourth larval instar (Korta et al. 2012). In stark contrast, *rsks-1* is dispensable for progenitor proliferation in adults (Korta et al. 2012), and AMPK mutants are still competent to undergo GSC quiescence in response to starvation (Seidel and Kimble 2015). The mechanisms controlling the response of adult *C. elegans* proliferative germ cells to diet remain largely unknown, and appear to be distinct from those controlling adult female *Drosophila* GSCs.

TOR is also required for the proliferation, growth and survival of differentiating progeny of *Drosophila* female GSCs (LaFever et al. 2010). *Tor* mutant dividing germline cysts in mosaic germaria show increased death, and follicles containing *Tor* mutant cysts grow at a markedly decreased rate (LaFever et al. 2010). Conversely, follicles carrying homozygous mutant cysts for *Tsc1*, an upstream inhibitor of TOR, grow significantly faster (LaFever et al. 2010). Under amino acid starvation, TORC1 inhibition by Nitrogen permease regulator like 2 and 3 (Nprl2 and Nprl3) protects pre-vitellogenic follicles from apoptosis, and knockdown of *nprl2* and *nprl3* in the germline prevents recovery of oogenesis following amino acid starvation (Wei and Lilly 2014). Mutations in follicle cells, in addition to affecting follicle cell growth itself, can also non-autonomously influence the growth of the underlying wild type germline, with *Tor* and *Tsc1* mutant follicle cells slowing down or accelerating follicle growth, respectively (LaFever et al. 2010). AMPK activity also controls

follicle cells, and *AMPK* mutant follicle cells are larger than wild type cells (Haack et al. 2013). Follicles carrying *Tor* mutant cysts eventually arrest at or prior to vitellogenesis, depending on allele strength, and degenerate (Pritchett and McCall 2012, LaFever et al. 2010). In another dipteran species, the yellow fever mosquito *Aedes aegypti*, where ovarian follicles remain in a previtellogenesis arrest until blood ingestion (Attardo et al. 2005), entry into vitellogenesis is also TOR-dependent, suggesting evolutionary conservation. Specifically, ovarian TORC1 activity is stimulated by a blood meal (Hansen et al. 2005, Roy and Raikhel 2012), and global knockdown of the TOR downstream effector ribosomal protein S6 kinase (S6K) inhibits yolk deposition (Hansen et al. 2005). Finally, TORC1 activity also regulates meiotic entry in the *Drosophila* ovary, as *Tor* mutant germline clones enter meiosis prematurely before 16-cell cysts are formed and the amino acid sensing GATOR1 complex promotes meiotic entry in the early germline by inhibiting TOR activity. Since global amino acid deprivation is not a condition of meiotic entry in the *Drosophila* ovary, however, GATOR1 may be acting in a nutrient-independent role (Wei et al. 2014)

Optimal levels of mTOR activity are also required in the mouse testis. TOR inhibition by rapamycin in neonatal mice reduces testis size, likely due to decreased germ cell proliferation and a block to meiosis (Busada et al. 2015). On the other hand, activation of mTORC1 as a result of global mutation of *promyeolocytic leukemia zinc finger* (*Plzf*) leads to compromised GDNF signaling and progressive germ cell loss, and these defects are rescued by rapamycin feeding (Hobbs et al. 2010). Nutrient-sensing pathways in Sertoli cells also influence the germline. For example, Sertoli cell-specific overactivation of TOR via *Tsc1* or *Tsc2* deletion or through decreased AMPK activity by conditional *Lkb1* knockout leads to progressive germ cell loss and loss of Sertoli cell quiescence (Tanwar et al. 2012). These

studies suggest that tight regulation of TOR is a recurring theme in the regulation of GSC lineages. Intriguingly, men with Peutz-Jeghers syndrome, frequently associated with *LKB1* mutations (Hemminkl 1998), are at risk for germ cell loss and Sertoli cell tumors, suggesting a conserved role for LKB1 in the human testis (Venara et al. 2001, Gourgari et al. 2012).

Diet-dependent hormones affect GSC lineages

Long-range, diet-dependent signals are integral components of the control of GSC lineages. As discussed below, hormones can regulate GSC lineages through direct actions on the germline itself or indirectly, via the niche or other somatic support tissues. Crosstalk among different hormonal systems and their integration within specific cellular contexts can further refine downstream responses.

Activation of insulin/insulin-like signaling is a highly conserved hormonal response to nutrient availability, and it can also affect downstream production of other hormones, such as the *Drosophila* steroid hormone 20-hydroxyecdysone (20E). In mammals, insulin secreted by pancreatic β cells in response to stimulation by glucose or amino acids signals through the insulin receptor (InR) (Figure 1.2). Insulin-like growth factors 1 and 2 (IGF1 and IGF2) are primarily synthesized in the liver and act through their receptors IGFR-1 and IGFR-2 to control cell growth (Siddle 2011). Activation of InR/IGFR receptors leads to multiple downstream events, including increased phosphoinositide-3 kinase (PI3K) activity and downstream phosphorylation and inhibition of the transcriptional factor FOXO. The *Drosophila* genome encodes eight insulin-like peptides (ILPs), whereas *C. elegans* has as many as 40, but in both cases, single homologs of InR and other downstream effectors transduce these signals (Kannan and Fridell 2013, Murphy and Hu 2013). 20E, the major steroid hormone in *Drosophila*, is produced from dietary cholesterol or ergosterol. In

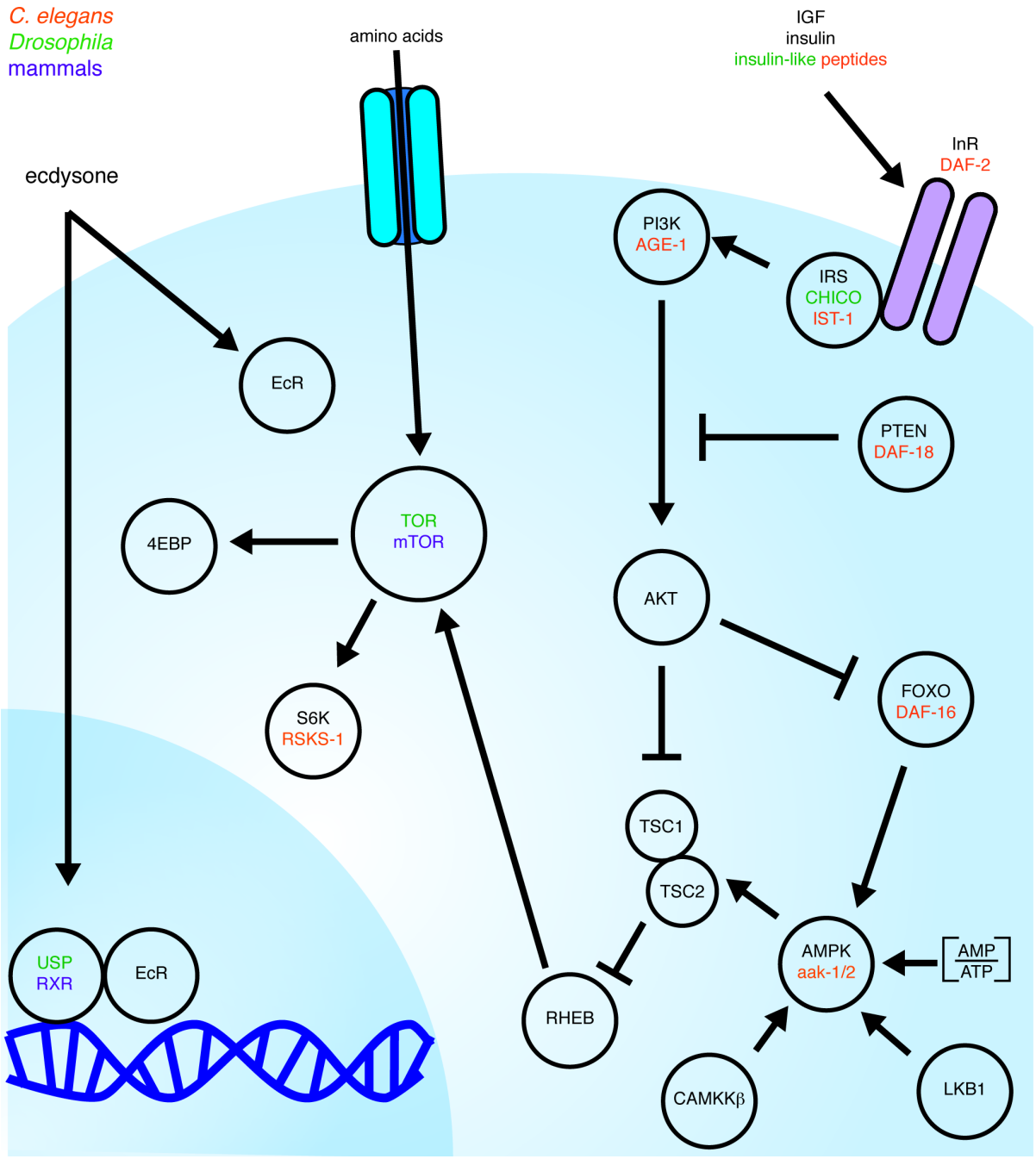


Figure 1.2. Conserved nutrient sensing pathways. Cells respond to intrinsic energy and nutrient levels and external stimulation by hormones to activate an interdependent, conserved cellular response to diet. Species-specific protein names are indicated in orange (*C. elegans*), green (*Drosophila*), and purple (mammalian); common names are shown in black.

females, late stage egg chambers produce 20E in a diet and insulin-dependent manner, whereas the source of the much lower titers of 20E in male hemolymph remains unidentified (Schwedes and Carney 2012). 20E acts through a nuclear hormone receptor composed of the ecdysone receptor [EcR, the homolog of farnesoid X receptor and liver X receptor (King-Jones and Thummel 2005)] and Ultraspiracle (Usp, the homolog of mammalian retinoid X receptor, RXR) to induce a wide range of downstream direct and indirect targets, including the early response genes *E74*, *E75*, and *broad* (Uryu et al. 2015).

ILPs directly stimulate GSC proliferation in *Drosophila*. In females, neural ILPs act directly on the germ line to control the G2 phase of the GSC cell cycle via PI3K but independently of dFOXO (LaFever and Drummond-Barbosa 2005, Hsu et al. 2008). While down-regulation of the insulin pathway preferentially extends G2, in females on a poor diet, both the G1 and G2 phases of the GSC division cycle are lengthened, suggesting the existence of a yet unknown diet-dependent signal that regulates G1 (Hsu et al. 2008). In male GSCs, germline-specific knockdown or inhibition of insulin signaling increases centrosome misorientation by disrupting localization of Apc2, a cortical protein required for centrosome anchoring (Roth et al. 2012). Conversely, constitutive activation of InR in the male germline rescues diet-induced centrosome misorientation. Further, an intact centrosome orientation checkpoint is required for the slow down of GSC division in males on a poor diet (Roth et al. 2012), suggesting a central role for this mechanism in the testis.

These studies in *Drosophila* are in contrast to findings in *C. elegans*. Although larval germ cells or adult germ cell tumors require insulin signaling for proliferation, normal adult germ cells proliferate in an insulin-independent manner (Michaelson et al. , Hubbard 2011, Dillin et al. 2002). Nevertheless, in adult *C. elegans*, insulin signaling couples nutrient

availability to oocyte development and progression through meiosis I through the activation of MAPK/ERK signaling (and, notably, independent of FOXO/DAF-16) (Lopez et al. 2013), indicating that germ cells in different developmental stages use distinct branches of insulin signaling.

Drosophila GSC maintenance also requires insulin signaling. In females, the mechanisms involved are clearly distinct from those controlling proliferation. Insulin signaling is not required in GSCs themselves for their maintenance. Instead, ILPs act directly on cap cells to promote Notch signaling, which is required for cap cell maintenance (Song et al. 2007), through FOXO inhibition; under low insulin signaling, FOXO induces high levels of the glycosyltransferase Fringe, leading to inhibition of the Notch receptor (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, Yang et al. 2013). In addition, ILPs directly stimulate the physical association between cap cells and GSCs through E-cadherin, independently of Notch signaling (Hsu and Drummond-Barbosa 2009). By contrast, in males, insulin signaling is intrinsically required in GSCs for their maintenance, as indicated by the higher frequency of loss of homozygous mutant *InR* GSCs in mosaic testes. Full rescue of GSCs loss induced by starvation, however, requires the constitutive activation of InR in both the germline and hub cells, suggesting that insulin signaling in the niche may also contribute to GSC maintenance (McLeod et al. 2010). These studies imply that conserved signaling pathways regulating similar processes (e.g. GSC proliferation or maintenance) can evolve distinct mechanisms to achieve that goal even between the different sexes of a given species.

20E directly controls GSC proliferation and maintenance in the *Drosophila* ovary, and it also has additional roles in ovarian and testicular somatic cells. Temperature-sensitive *EcR*

mutants show increased loss and reduced proliferation rates of GSCs, and both of these phenotypes reflect an intrinsic requirement for ecdysone signaling in GSCs, based on genetic mosaic analyses (Ables and Drummond-Barbosa 2010). Ecdysone signaling controls GSCs independently of insulin signaling by modulating the responsiveness of GSCs to BMP ligands from the niche. This role requires the downstream target *E74* [a member of the *ets* proto-oncogene family (Burtis et al. 1990, Karim et al. 1990)] specifically, as GSCs mutant for *usp* or *E74*, but not *E75* or *broad*, show low levels of a BMP signaling reporter and are rapidly lost from the niche (Ables and Drummond-Barbosa 2010). *E75* [a homolog of mammalian *peroxisome proliferator-activated receptor γ* (*PPARγ*) (King-Jones and Thummel 2005)] is also required in escort cells for GSC maintenance, and expression of a dominant negative form of EcR in escort cells disrupts female germ cell differentiation (Konig et al. 2011, Morris and Spradling 2012), underscoring the complexity of 20E regulation of the early female germ line. In later follicle development, *E78*, an *E75*-related gene that is also an early ecdysone target (Stone and Thummel 1993), is cell autonomously required for germline survival and functionally interacts with ecdysone signaling (Ables et al. 2015). Many additional targets of ecdysone contribute to its various roles in the female GSC lineage, although more in depth analysis is needed (Ables submitted). In the testis, EcR knockdown in the somatic lineage leads to CySC and GSC loss, and death of differentiating germ cells, although the specific mechanisms involved remain unclear (Li et al. 2014).

Progression through vitellogenesis is a major nutritional checkpoint in *Drosophila* and other insects. *Drosophila* temperature-sensitive *EcR* mutants and *E75* mutant germline clones fail to progress through vitellogenesis (Carney and Bender 2000, Buszczak et al. 1999), indicating a germline requirement for ecdysone signaling. *Drosophila InR* global mutants are

defective in ecdysteroid production and vitellogenesis, and these defects appear to be at least partially rescued by treatment with a juvenile hormone analog (Tu et al. 2002). Follicles containing germline clones that are mutant for *InR* or defective for PI3K signaling, however, have reduced rates of growth and blocked vitellogenesis, clearly indicating that insulin signaling through PI3K is required in the germline itself for these processes (Hsu et al. 2008, LaFever and Drummond-Barbosa 2005). Unlike for GSC proliferation, however, insulin signaling does not involve FOXO and, instead, feeds into TOR signaling for the control of follicle growth (Hsu et al. 2008). Interestingly, insulin/TOR signaling in follicle cells is required for the processing body and microtubule rearrangements that occur in the underlying germ line in response to diet (Burn et al. 2015). In *A. aegypti*, vitellogenesis also requires insulin signaling (Brown et al. 2008, Gulia-Nuss et al. 2011), and the ovary expresses EcR and shows induction of ecdysone response genes, including E75, upon blood feeding (Cho et al. 1995, Swevers 2009, Pierceall et al. 1999), suggesting conserved mechanisms of vitellogenesis control. After vitellogenesis in the *Drosophila* ovary, a reduction in insulin signaling induces a metabolic shift toward glycogen storage and mitochondrial quiescence, preparing the oocyte for fertilization (Sieber et al. 2016).

Multiple hormones also regulate mammalian GSC lineages, although the connection to diet is not always well understood. Retinoic acid (RA) is derived from dietary vitamin A and signals through retinoic acid receptors (RARs) and their partners, RXRs (Blomhoff and Blomhoff 2006). The multiple isoforms of RAR and RXR are differentially expressed, lending an additional level of specificity to RA signaling. Analogously to ecdysone signaling in *Drosophila*, RA signaling is required in both the germline and somatic support cells of the mouse testis (Hogarth and Griswold 2013). Knockout of all three *RAR* or *RXR* isoforms

specifically in the germ line suppressed spermatogonia proliferation, consistent with the role of vitamin A in maintaining spermatogenesis (Gely-Pernot et al. 2012, Ghyselinck et al. 2006). Furthermore, Sertoli cell-specific knockout of retinaldehyde dehydrogenases, which are required for RA synthesis, blocks sperm meiotic entry (Tong et al. 2013, Raverdeau et al. 2012). Testicular RA titers are also reduced in mice genetically depleted of macrophages, resulting in spermatogonial differentiation defects (DeFalco et al. 2015). Conversely, ectopic RA administration induces differentiation and meiotic entry in specific, poised subsets of spermatogonia (Endo et al. 2015). While spermatogonia can directly respond to RA *in vivo* (Zhou et al. 2008), it is unlikely that RAR/RXR are the sole mediators of its activity. For example, spermatogonia lacking all three isoforms of either RAR or RXR can still enter meiosis, albeit at a low frequency (Gely-Pernot et al. 2015), hinting that RA could act independently of the nuclear hormone receptor dimer or act indirectly through other testicular cell types (Gely-Pernot et al. 2015). Indeed, Sertoli cell-specific *RARα* knockout in *RAR*γ mutant mice have a complete block to meiosis, suggesting that the RA-dependent meiosis signal requires paracrine signaling from Sertoli cells (Gely-Pernot et al. 2015). While spermatogonial RAR and RXR isoforms are dispensable for germ cell survival (Gely-Pernot et al. 2015), Sertoli cell RARα promotes germ cell survival in aging animals (Vernet et al. 2006). Sertoli cell-specific knockout of *RAR*α leads to progressive testis deterioration, with death of spermatocytes and spermatids (Vernet et al. 2006). Intriguingly, this phenotype is not recapitulated in Sertoli cell knockout of all isoforms of RXR, invoking again an RXR-independent RA signaling mechanism. Bioinformatic analysis further suggests that several genes responsible for establishing tight junctions to create the blood testis barrier contain

retinoic acid response elements, suggesting that RAR activity in Sertoli cells may regulate the integrity of the blood-testis barrier (Chung et al. 2010).

The hypothalamic-pituitary-gonadal axis is a major regulator of mammalian spermatogenesis, and there is evidence to suggest that diet and obesity can impact its function. Gonadotropin releasing hormone (GnRH) secreted from the brain induces secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary, which act on Sertoli and Leydig cells, respectively, to control SSC lineage activity (O'Shaughnessy 2014). LH induces testosterone production by Leydig cells, and testosterone signals via the androgen receptor (AR), a nuclear hormone receptor predominantly expressed in Sertoli cells (Sar et al. 1990, reviewed in Walker and Cheng 2005), although recent evidence suggests the presence of a functional AR in human sperm (Aquila et al. 2007). Aromatase activity in the testis, and to a lesser extent in peripheral tissues, catalyzes the synthesis of estradiol, the most potent biological estrogen, from androgens (Marcus 1976). Estrogens are integral regulators of spermatogenesis, in part because they feed back on the brain to regulate LH and FSH secretion (Simpson et al. 1999, Simpson et al. 2000). While our current understanding of nutrient inputs to the hypothalamic-pituitary-gonadal axis is complicated by the tissue dysfunction associated with its disruption, studies in rats suggest that GnRH release decreases in fasted animals (Gruenewald and Matsumoto 1993). Administration of insulin to female volunteers produces a serum pulse of LH, consistent with insulin acting on the GnRH neurons (Moret et al. 2009). Conflicting reports in mouse models, however, debate whether insulin acts directly on GnRH neurons (Evans et al. 2014, DiVall et al. 2015), and the response to insulin could be sexually dimorphic (Kovacs et al. 2002, Castellano et al. 2006). Furthermore, Leydig cell testosterone production is perturbed

in mice with Sertoli cell-specific *InR* or *Igf1r* knockout, suggesting that diet-dependent signaling controls the hypothalamic-pituitary-gonadal axis at the testis level (Pitetti et al. 2013). *Igf1* knockout mice also have reduced spermatogenesis, although it is unclear whether there is a developmental contribution to this phenotype (Baker et al. 1996). Metabolic state influences the hypothalamic-pituitary-gonadal axis in adult men and women. Women with diet-induced amenorrhea have reduced plasma levels of estrogen, FSH, and LH (Couzinet et al. 1999), and high aromatase expression in post-menopausal obese women leads to more circulating estrogen (Baglietto et al. 2009). Additionally, men with type 2 diabetes or obesity are more likely to be testosterone deficient, further suggesting a possible connection between the hypothalamic-pituitary-gonadal axis and diet (Kelly and Jones 2013, Wang et al. 2011).

The hypothalamic-gonadal-pituitary axis regulates both the germline and somatic support tissues in the mammalian testis. Testosterone and FSH act semi-redundantly through their receptors (AR and FSHR, respectively) on Sertoli cells to promote germ cell survival (Walker and Cheng 2005). Estrogen signaling is also critical for germline survival during spermatogenesis, as shown in a knock-in ER mutant mouse that does not respond to estrogen (Sinkevicius et al. 2008, Sinkevicius et al. 2009). In accordance, global knockout of aromatase in mice results in increased germ cell apoptosis in the testis (Robertson et al. 1999). In both estrogen-signaling incompetent and aromatase mutant mice, circulating LH and FSH levels were slightly higher and unchanged, respectively, suggesting that germ cell apoptosis is not simply a downstream effect of estrogens controlling LH and FSH secretion (Sinkevicius et al. 2009, Robertson et al. 1999, Simpson et al. , Simpson et al. 2000). FSH is required for testis growth in both mice and humans (Kumar et al. 1997, Phillip et al. 1998), and *FSHR* knockout mice have impaired spermatid elongation (Krishnamurthy et al. 2000).

AR is absolutely required in Sertoli cells for completion of meiosis (Abel et al. 2008, De Gendt et al. 2004). Furthermore, mice with a conditional knockout of AR in peritubular myoid cells have significantly reduced sperm counts, suggesting multiple sites of action for testosterone (Zhang et al. 2006).

Additional inter-organ communication influences GSC lineages

Beyond the more "traditional" hormone examples discussed above, the extent of GSC lineage regulation by signals originating in other organs, with distinct organs providing various types of information, is just beginning to be appreciated. The molecular mechanisms of many of these signaling axes remain unknown, but proteohormones, signaling lipids or metabolites, and mobilized nutrients from one organ to another are likely participants.

The nervous system regulates physiological circuits that marry inputs from the external environment, including diet, to whole-body physiology either through changes in organismal behavior or more directly. As discussed above, *Drosophila* ILPs are diet-dependent neuropeptides that directly regulate GSC lineages and nearby somatic support cells (Hsu and Drummond-Barbosa 2009, LaFever and Drummond-Barbosa 2005). Ecdysone produced by ovarian follicles acts on the brain to promote female-specific feeding behavior, increasing nutrient uptake and supporting oogenesis (Sieber and Spradling 2015). Octopaminergic neurons innervate the ovaries and reproductive tract and are essential for *Drosophila* ovulation (Lee et al. 2003, Monastirioti 2003, Deady and Sun 2015). Interestingly, a subset of octopaminergic neurons becomes hyperactive under starvation (and promotes foraging behavior) (Yang et al. 2015), suggesting a potential molecular link between ovulation and nutrient availability. During *C. elegans* development, transforming growth factor β

(TGFβ/DAF-7) expression in chemosensory neurons is activated by food availability and low dauer pheromone, which is involved in sensing population density (Ren et al. 1996, Schackwitz 1996, Dalfo et al. 2012). *daf-7* mutant worms enter meiosis prematurely, thereby reducing progenitor number, and downstream signaling pathway components are required in the DTC, indicating a niche-mediated role for TGFβ in maintaining a large germline progenitor pool from which gametes can be generated (Dalfo et al. 2012). As mentioned earlier, GnRH released from the mammalian hypothalamus stimulates pituitary FSH and LH release, which in turn act on testis somatic cells to control the germline (O'Shaughnessy 2014). In adult men, even short periods of fasting can suppress GnRH, leading ultimately to a fall in LH-induced testosterone production (Trumble et al. 2010). Thus, nutritional cues may be transmitted to the germline via the brain in multiple organisms, although specific strategies can vary.

GSC lineages are also controlled by endocrine signals from other organs, including the adipose tissue. Accordingly, obese men are more likely to be subfertile (Martin 2014, Kawwass et al. 2015), and diet-induced obesity in rats is linked to decreased sperm motility (Fernandez et al. 2011, Palmer et al. 2012). Mammalian proteohormones secreted from adipocytes, or adipokines, modulate homeostasis by regulating multiple processes (Cao 2014). For example, leptin signals satiety to the brain and controls metabolism in peripheral tissues (Moran and Phillip 2003), and adiponectin causes pleiotropic effects in peripheral tissues, sensitizing them to insulin (Yamauchi and Kadowaki 2013) and preventing inflammation (Fantuzzi 2008) and apoptosis (Shibata et al. 2005). While adiponectin is produced primarily in adipocytes (Maeda et al. 1996), its circulating levels are reduced in obese individuals, and genetically-defined circulating adiponectin levels can influence insulin

sensitivity in humans (Gao et al. 2013, Kizer 2013). In mammals, adiponectin forms various, stable multimers, from trimers to high molecular weight forms (Schraw et al. 2008); multimers do not appear to freely interconvert, and their presence is sexually dimorphic in humans (Peake et al. 2005). Adiponectin signals via two differentially expressed receptors, AdipoR1 and 2 (Yamauchi et al. 2003), inducing a wide range of downstream effectors (Turer and Scherer 2012). Multimeric adiponectin also binds and signals via T-cadherin (Hug et al. 2004, Denzel et al. 2010, Parker-Duffen et al. 2013), although the mechanism of signal transduction remains unknown. Adiponectin signaling regulation could occur at multiple levels. First, several transcription factors, including PPARγ, promote adiponectin expression (Liu and Liu 2010), and adipocyte-specific deletion of PPARγ lowers the circulating levels of adiponectin (He et al. 2003). Multimer assembly (Liu et al. 2008) and vesicular packaging (Xie et al. 2006) are potential additional points of regulation. How does adiponectin signaling exert tissue-specific effects? It is likely that cell-specific regulation of its many downstream effectors, including the nutrient-sensing pathways TOR (Barb et al. 2007) and AMPK (Yamauchi et al. 2002), contribute to these precise responses. Some studies suggest that the pleiotropic effects of adiponectin can be ascribed to ceramidase activation (Holland et al. 2011), in itself a constituent of a major signaling network (Hannun and Obeid 2008). The ever-growing collection of adiponectin signaling literature indicates that these complex questions require further investigation.

Several lines of evidence suggest that adipokines might influence reproduction, although reports are conflicting (Kawwass et al. 2015). AdipoR1 and 2 are expressed in the human and mouse hypothalamus (Dupont et al. 2014), and their transcripts are detected in Leydig cells and the testicular epithelium of rats (Caminos et al. 2008). Adiponectin

knockout mice, however, are fertile, while mice lacking AdipoR2 do not produce sperm (Bjursell et al. 2007), suggesting possible adiponectin-independent roles for this receptor. The leptin receptor is also expressed in the mammalian testis (Landry et al. 2013), and leptin-deficient mice have increased germ cell death and fertility defects (Mounzih et al. 1997, Bhat et al. 2006). Conversely, spermatogenesis defects in men show an association with increased expression of leptin and its receptor in the testis (Ishikawa et al. 2007). Deletion of leptin receptor specifically in the hypothalamus recapitulates many of the phenotypes of leptin-deficient mice, however, suggesting that leptin acts via the brain to control fertility (Ahima et al. 2006). Although these studies are important steps towards our understanding of how mammalian adipokines influence the germline (Kawwass et al. 2015), much remains to be learned about how these and other adipocyte factors control reproduction.

Adipocyte factors also contribute to the control of GSC lineages in invertebrate model systems. In *Drosophila*, the fat body, an organ composed of adipocytes and hepatocyte-like oenocytes (Gutierrez et al. 2007), has endocrine roles that control development, metabolism and behavior (Arrese and Soulages 2010). Although *C. elegans* lack a distinct fat storage organ, dedicated lipid storage cells are found in the intestine and epidermis (Mullaney and Ashrafi 2009). Adipokine signaling modules are conserved in *Drosophila* and *C. elegans*, and they influence the GSC lineages of these organisms (Rajan and Perrimon 2012, Laws et al. 2015, Kwak et al. 2013, Svensson et al. 2011). The sole adiponectin receptor in *Drosophila*, AdipoR, is intrinsically required by ovarian GSCs for their maintenance (Laws et al. 2015). In *C. elegans*, deletion of adiponectin receptor homologs *paqr-1,2,* and *3* causes extensive defects, including reduced brood size (Svensson et al. 2011). Adiponectin-like ligands have not yet been identified in *C. elegans* or *Drosophila*; nevertheless, *ex vivo*

cultures of fly larval brains respond to stimulation by recombinant mammalian adiponectin (Kwak et al. 2013), suggesting that the *Drosophila* receptor recognizes an endogenous ligand with conserved three-dimensional structure. Additionally, Unpaired 2 (Upd2) is secreted from *Drosophila* adipocytes and stimulates brain ILP secretion (Rajan and Perrimon 2012), presumably indirectly affecting oogenesis. Human leptin transgenic expression or feeding rescues the *upd2* mutant phenotype, suggesting that Upd2 is the functional equivalent of leptin despite lack of primary sequence homology (Rajan and Perrimon 2012).

Studies in *Drosophila* also show that adipocytes play an important role in reproduction by transmitting nutritional information to the ovary. In *Drosophila* females, a slight decrease in amino acid levels within adult adipocytes through the knockdown of single amino acid transporters significantly increases the rate of GSC loss from the niche and partially blocks ovulation through distinct mechanisms (Armstrong et al. 2014). Low amino acid levels trigger the evolutionarily conserved amino acid response pathway through unloaded tRNA-mediated activation of the GCN2 kinase within adipocytes to cause GSC loss, whereas amino acids modulate TOR to regulate oocyte ovulation (Armstrong et al. 2014). Adipocyte factors acting downstream of the amino acid response pathway or TOR to control the GSC lineage, however, remain unidentified. In *A. aegypti*, global knockdown of amino acid transporters reduces egg laying (Carpenter et al. 2012), and TOR activity in the fat body is induced after blood feeding (Hansen et al. 2005) and required for ovarian follicle vitellogenesis (Hansen et al. 2004, Hansen et al. 2005, Roy and Raikhel 2012, Roy and Raikhel 2011, Carpenter et al. 2012). Fat body transcription of *vitellogenin*, which is physiologically triggered by 20E following a blood meal, can be induced by *ex vivo* treatment of cultured fat bodies with amino acids, but this effect is suppressed by TOR inhibition (Hansen et al. 2004, Hansen et

al. 2005). *In vivo*, knockdown of components of the TOR pathway reduces egg laying and can impair yolk uptake and egg viability (Hansen et al. 2004, Hansen et al. 2005), although these effects likely reflect potential roles of TOR in multiple locations, as is the case in *Drosophila*.

Many additional organs are important regulators of physiology; therefore, it would be logical to explore their potential roles in contributing to the control of GSC lineages. For example, nutrients are absorbed at the intestine, and in *Drosophila* females, feeding conditions change the physiology of the midgut (Cognigni et al. 2011). Mating also leads to extensive remodeling of the midgut and increased lipid metabolism, and these changes are required for normal levels of fecundity (Reiff et al. 2015). Although some of these effects likely reflect changes in efficiency of digestion and nutrient absorption, it is conceivable that more active signaling occurs between the intestine and GSC lineages. It is also important to consider the effect of the gut microbiome in reproduction. The type of bacteria ingested by *C. elegans* influences its brood size (Yu et al. 2015). Also, in the absence of the nuclear hormone receptor *nhr-114* (the homolog of HNF4), worms are sterile when fed a specific strain of bacteria (Gracida and Eckmann 2013). *nhr-114* activity is detected in both the germline and gut, but it does not appear to be required in the germline. Tryptophan supplementation rescues this phenotype, suggesting that *nhr-114* may help buffer dietary changes in the gut (Gracida and Eckmann 2013). Interestingly, germ-free mice have impaired blood-testis barrier and lumen formation in seminiferous tubules, and reduced levels of serum LH and FSH and testicular testosterone. Exposure of these mice to a strain of bacteria that secrete high levels of the short-chain fatty acid butyrate restored integrity of the blood-testis barrier (Al-Asmakh et al. 2014). Recent studies have also shown that muscles secrete

Figure 1.3. Cell type-specific requirements for diet-dependent pathways involved in the control of GSCs and their progeny. (A) In the *Drosophila* ovary, intrinsic, local, and tissue non-autonomous signals coordinately regulate the GSC lineage. In addition to the niche, the brain, follicle cells, and adipocytes all communicate with the GSC lineage as part of the response to diet. **(B)** GSCs in the *Drosophila* testis require InR intrinsically for maintenance and proliferation. EcR in cyst stem cells (CySCs) promotes GSC maintenance and the survival of their progeny. **(C)** In the developing *C. elegans* gonad, but not in the adult, proliferation is regulated intrinsically by insulin, TOR, and AMPK (AAK-1/2). TGFβ ligand (DAF-7) promotes progenitor pool maintenance via the distal tip cell (DTC). **(D)** The mouse SSC pool is regulated by Leydig, Sertoli, and peritubular myeloid (PTM) cells, as well as macrophages. An additional layer of control is provided by the hypothalamic-pituitary-gonad axis, which controls the activity of these somatic support cells to influence spermatogenesis. Biological processes are indicated in green and cell types in gray. For details, see text and Table 1.1.

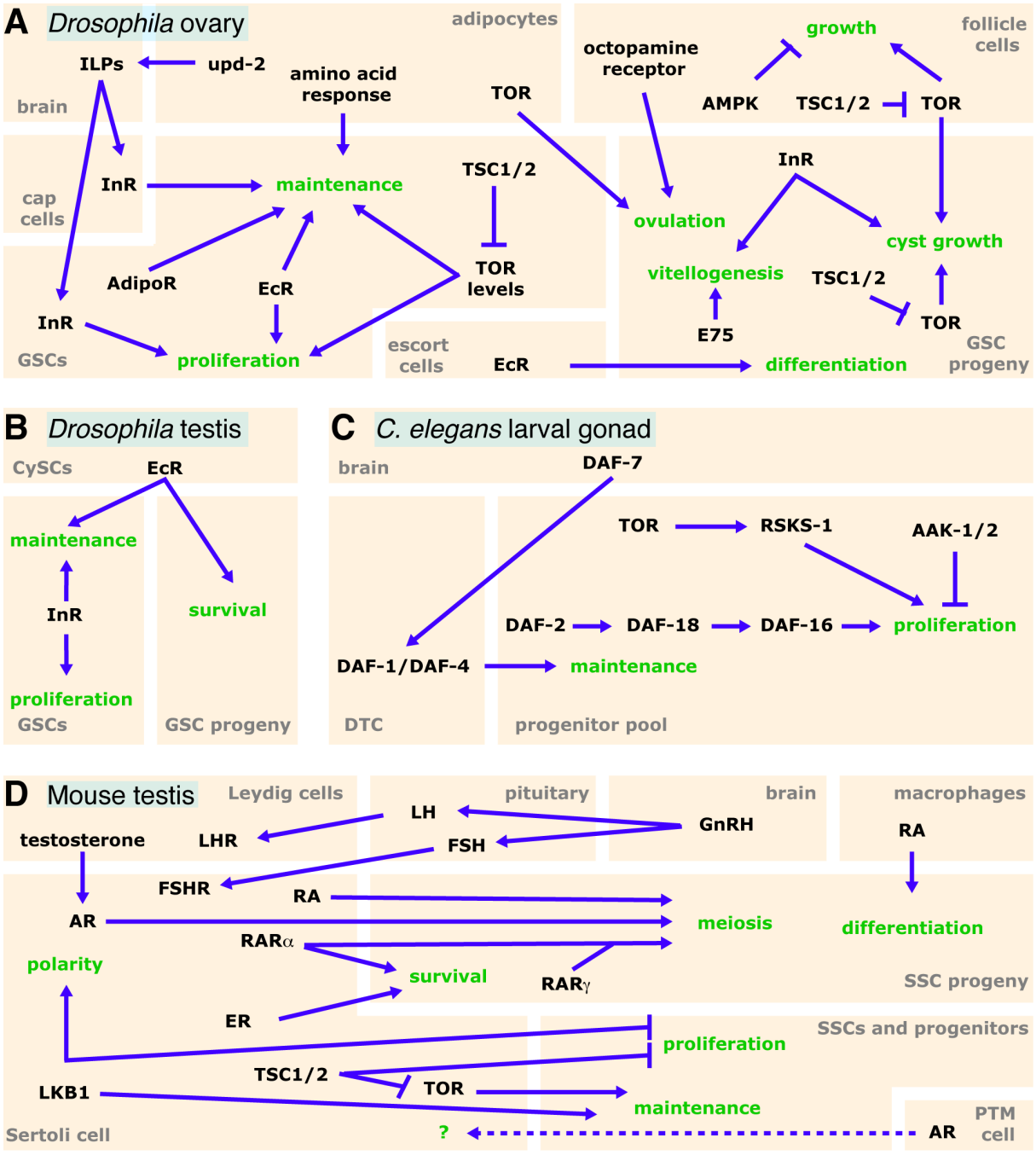

A Drosophila ovary
adipocytes
ILPs
upd-2
brain
amino acid response
TOR
octopamine receptor
growth
follicle cells
AMPK
TSC1/2
TOR
cap cells
InR
maintenance
TSC1/2
ovulation
InR
cyst growth
AdipoR
EcR
TOR levels
vitellogenesis
TSC1/2
InR
GSCs
proliferation
escort cells
EcR
E75
TOR
differentiation
GSC progeny
B Drosophila testis
CySCs
EcR
maintenance
InR
survival
proliferation
GSCs
GSC progeny
C C. elegans larval gonad
brain
DAF-7
TOR
RSKS-1
AAK-1/2
DAF-2
DAF-18
DAF-16
proliferation
DAF-1/DAF-4
maintenance
DTC
progenitor pool
D Mouse testis
Leydig cells
pituitary
brain
macrophages
LH
GnRH
RA
testosterone
LHR
FSH
FSHR
RA
AR
meiosis
differentiation
RARα
polarity
survival
RARγ
SSC progeny
ER
proliferation
SSCs and progenitors
TSC1/2
TOR
LKB1
maintenance
Sertoli cell
?
AR
PTM cell

Table 1.1. Physiological regulation of GSC lineages by diet-dependent pathways.

Pathway	Organism	Role
AMPK	*C. elegans*	Inhibits germline proliferation during larval starvation (Fukuyama et al. 2012, Narbonne and Roy 2006)
	Drosophila	Inhibits follicle cell growth in the ovary (Haack et al. 2013)
	mouse	Sertoli cell LKB1 promotes SSC proliferation and maintenance (Tanwar et al. 2012)
TOR	*C. elegans*	Promotes larval progenitor proliferation (Korta et al. 2012)
	Drosophila	Levels control GSC maintenance and proliferation (LaFever et al. 2010, Sun et al. 2010) and germline cyst survival (LaFever et al. 2010) in the ovary
		Regulates ovarian cyst growth intrinsically and via follicle cells (LaFever et al. 2010, Sun et al.)
	A. aegypti	Elevated in ovary following blood meal (Hansen et al. 2005, Roy and Raikhel 2012)
		Required in the fat body for vitellogenesis (Hansen et al. 2004, Hansen et al. 2005, Roy and Raikhel 2012, Roy and Raikhel 2011, Carpenter et al. 2012)
	mouse (*mTOR*)	Promotes germline proliferation and meiosis during development (Busada et al. 2015)
		Global hyperactivation inhibits SSC maintenance (Hobbs et al. 2010)
		Overactivation in Sertoli cells leads to intrinsic polarity defects, reduced SSC proliferation, and SSC loss (Tanwar et al. 2012)
Insulin	*C. elegans*	Promotes PGC proliferation (Michaelson et al. 2010)
	Drosophila	Promotes ovarian GSC proliferation, cyst growth, and vitellogenesis (LaFever and Drummond-Barbosa 2005)
		Controls ovarian GSC maintenance via cap cells (Hsu and Drummond-Barbosa 2009)
		Promotes GSC proliferation and maintenance in testis (Roth et al. 2012, McLeod et al. 2010)
	A. aegypti	Required for progression through vitellogenesis (reviewed in Attardo et al. 2005)
	mouse	Global requirement for *InR* and *Igf1r* for testicular development (Pitetti et al. 2013)
		InR and *Igf1r* promote developmental Sertoli cell proliferation (Pitetti et al. 2013)
		Global *IRS2* mice have small testes and progressive germ cell loss as adults (Griffeth et al. 2013)
		Global *Igf1* mice have reduced spermatogenesis (Baker et al. 1996)
Ecdysone	*Drosophila*	Promotes ovarian GSC maintenance, proliferation (Ables and Drummond-Barbosa 2010), and vitellogenesis (Buszczak et al. 1999)
		Required in escort cells for germline differentiation (Konig et al. 2011, Morris and Spradling 2012)
		Required in CySCs for GSC maintenance and progeny survival in the testis (Li et al. 2014)
	A. aegypti	After a blood meal, ecdysone response genes expressed in ovary (Pierceall et al. 1999)
		Required in the fat body for vitellogenesis[a] (Martin et al. 2001)
Retinoic acid	mouse	Germ cell RARα and Sertoli cell RARγ are together required for meiosis (Gely-Pernot et al. 2015)
		Required in Sertoli cells for germ cell meiosis (Tong et al. 2013, Raverdeau et al. 2012)
Androgen receptor	mouse	Required in Sertoli cells for cell survival and meiosis (Abel et al. 2008, Hobbs et al. 2010, De Gendt et al. 2004)
		Required intrinsically by PTMs for normal sperm counts (Zhang et al. 2006)
AdipoR	*C. elegans*	Global mutation reduces brood size (Svensson et al. 2011)
	Drosophila	Required for ovarian GSC maintenance(Laws et al. 2015)
	mouse	*AdipoR2* global mutants are aspermic (Bjursell et al. 2007)
Leptin	*Drosophila*	Required in fat body to promote ILP secretion from the brain (Rajan and Perrimon 2012)
	mouse	Promotes fertility (Mounzih et al. 1997) and germ cell survival (Bhat et al. 2006)

peptide hormones, or myokines, in *Drosophila* and mice (Demontis et al. 2013, Demontis and Perrimon 2010, Demontis et al. 2014), and genetic manipulations in muscles affect the physiology of the fly (Demontis and Perrimon 2010). In mice, osteocalcin secreted from bones modulates spermatogenesis by promoting testosterone production in Leydig cells (Oury et al. 2011). Further, in insects, sex peptides transferred during mating trigger a host of physiological changes, including many upstream of GSC lineage activity (Soller et al. 1997, Kubli 2003). In *C. elegans,* major sperm protein (MSP) released by sperm promotes oocyte growth, meiotic maturation and ovulation in proximal oocytes via several mechanisms (Miller et al. 2001, Harris et al. 2006, Govindan et al. 2009, Kim et al. 2013). Future studies should consider many possible modes of action for various signals coming from multiple organs in regulating reproductive lineages.

Discussion

As reviewed here, highly conserved diet dependent pathways control GSC lineages, revealing interesting similarities and differences in their specific roles in different contexts (Figure 1.3). As the diet-dependent molecular, cellular and physiological mechanisms controlling GSC lineages are further investigated in multiple models, common themes and more specific strategies shaped by evolution will become clearer. As this field advances, the continued use of tissue- and cell-type-specific manipulations in an *in vivo* setting will be crucial to understand the full range of contributions of any given hormone or other factor to the regulation of the germline.

Not surprisingly, many other factors besides diet can impact organismal physiology. For example, changes in germline activity accompany aging in multiple organisms (Tatar 2010, Oatley and Brinster 2012). *C. elegans* and *Drosophila* fecundity is impaired in older

females (Herndon et al. 2002, Partridge and Fowler 1992), sperm aneuploidy increases as mice age (Lowe et al. 1995), and sperm count declines in older men (Eskenazi et al. 2003). The germ line ages intrinsically and is also affected by the aging of the soma. In *C. elegans*, oocytes deteriorate as mated hermaphrodites age, and naturally occurring cell death protects oocyte quality in younger animals, as cell death mutants *ced*-3 and *ced*-4 have a premature drop in oocyte quality. This is a germline-autonomous effect because mutations that block only somatic cell death do not impair oocyte quality (Andux and Ellis 2008). In contrast, there is a systemic effect of aging in mouse SSCs, as transplantation of SSC from old donors to young recipients restores youthful function to the older SSCs (Ryu et al. 2006). In *Drosophila* males and females, GSCs proliferate more slowly as they age (Wallenfang et al. 2006, Cheng et al. 2008, Pan et al. 2007). By contrast, long-lived *methuselah* mutant males do not experience a drop-off in GSC proliferation as they age (Wallenfang et al. 2006). The number of niche cells and of GSCs decline with age in males and females (Wallenfang et al. 2006, Xie and Spradling 2000, Hsu and Drummond-Barbosa 2009, Zhao et al. 2008, Boyle et al. 2007). E-cadherin, and BMP and insulin signaling levels decline with age in the ovary (Pan et al. 2007, Hsu and Drummond-Barbosa 2009), and overexpression of E-cadherin or AdipoR in the germline (Pan et al. 2007, Laws et al. 2015) or Dpp or ILPs in the soma (Pan et al. 2007, Hsu and Drummond-Barbosa 2009) can reverse age-related GSC loss. Somatic and germline overexpression of superoxide dismutase (SOD), an antioxidant enzyme, rescues the age-associated decline of GSC function and cap cell number (Pan et al. 2007), further emphasizing the complexity of effects of aging on the germ line.

Disease states, including cancer, also disrupt organismal homeostasis. Tumors are their own dynamic signaling centers (Karagiannis et al. 2010) and can both hijack normal

metabolism to support their growth and secrete factors that have pleiotropic effects (Patel et al. 2014). For example, tumors induced in the midgut of adult *Drosophila* or transplanted into the fly hemocoel secrete the ILP binding protein IMP-L2 (Kwon et al. 2015, Figueroa-Clarevega and Bilder 2015), causing systemic wasting, including that of ovaries. In humans, this wasting, called cachexia, is a hallmark of end-stage cancer and is uncoupled from tumor burden (Petruzzelli and Wagner 2016, Fearon et al. 2012), underscoring the role of systemic factors in cancer pathologies. While our understanding of the effect of cachexia on mammalian GSC lineages is limited, cachetic patients are often insulin resistant (Honors and Kinzig 2012), and chronic inflammation, found in many cancers (Crusz and Balkwill 2015), can lead to Interleukin-6 mediated hyperactivation of the hypothalamic-gonadal-pituitary axis (Raber et al. 1997). Therefore, although it is clear that many cancer treatments can impair fertility (Suhag et al. 2015, Vakalopoulos et al. 2015), it is also possible that the physiological changes caused by the tumors themselves may also have effects on the germ line of patients.

Finally, there is also evidence that diet can alter the epigenetic state of the germ line and thereby impact the next generation. For example, male mice fathered by fasted males have lower serum glucose concentrations than those fathered by normally fed animals (Anderson et al. 2006). Adult offspring of *Drosophila* males fed a high sugar diet have increased food intake, higher adiposity, and defects in lipid mobilization, a metabolic signature dependent on specific subtypes of heterochromatin (Ost et al. 2014). Notably, similar genomic derepression patterns are predictive for obesity in mice and humans, indicating a conserved pathway of diet-induced phenotypic variability (Ost et al. 2014). While the effects of paternal diet lasted a single generation in *Drosophila* (Ost et al. 2014), in

certain mouse genetic backgrounds, paternal high fat diet causes offspring infertility in male and females for two generations (Fullston et al. 2012). Although the molecular mechanisms remain unknown, epidemiological studies have identified multi-generational ramifications for the children of males exposed to occupational and environmental toxins, including lead and pesticides, many of which could be mediated epigenetically (Soubry et al. 2014). As we learn more about how whole-body physiology control of GSC lineages, new light will be shed on how changes caused by diet, aging, diseases, infections, injuries or other stressors affect fertility and, potentially, future generations.

Outline and summary

The goal of this research is to investigate the physiological regulation of the *Drosophila* ovarian stem cell lineages by nutrient-dependent processes. While I primarily focused on ovary-intrinsic signaling, considering the downstream effectors of signals that impinge on the germline, I also contributed to work that characterized the coordination of nutrient responses in adult adipocytes with oogenesis. In Chapter II, I describe the major approach used in my research, FLP/FRT-mediated genetic mosaic analysis. In addition to circumventing developmental lethality, permitting the analysis of mutant cells in adult animals, this technique allows analysis of gene function at the single cell level. Understanding the requirements of different cells within the same tissue was critical for my investigation of AMPK and AdipoR function in the adult ovary, and will be integral in parsing the complex signaling networks that converge on that organ. In Chapter III, I present a published study of the AdipoR homolog in the *Drosophila* ovary. While adiponectin has been widely reported to sensitize tissues to insulin and has several reported roles in progenitor cells, whether those functions were related to each other was unclear.

Surprisingly, I found an insulin-independent role for AdipoR in controlling GSC maintenance, which should expand the scope of studies of the adiponectin signaling axis. Furthermore, I demonstrated that overexpression of AdipoR in the germline mitigates the progressive GSC loss that accompanies age, suggesting that a decline in adiponectin signaling could contribute to some aspects of GSC aging. In Chapter IV, I discuss ongoing studies of AMPK function in the germline and somatic stem cell lineages of the adult ovary. Interestingly, I identify both diet-dependent and -independent roles for AMPK, and distinct roles in the germline and somatic lineages, leading to questions about its downstream effectors in different cellular contexts. Chapter V is a published study describing one mechanism of adipocyte control of oogenesis. Together with Dr. Alissa Armstrong, I showed that knockdown of single amino acid transporters specifically in adult adipocytes controls GSC number over time in the *Drosophila* ovary. Amino acids in adipocytes modulate GSC maintenance independently of TOR, but through the amino acid response pathway, a cellular response triggered by high levels of unloaded tRNAs. Future studies in the soon-to-be-established Armstrong lab will follow up on these experiments to understand what signaling pathways function downstream of amino acid sensing in adipocytes. In Chapter VI, I present unpublished experiments I conducted over the course of my graduate career, all with an eye toward understanding the physiological environment that regulates GSC activity in the ovary. Finally, I discuss the implications of these studies in understanding the physiological control of germ lines and stem cell lineages more generally.

CHAPTER II

GENETIC MOSAIC ANALYSIS OF STEM CELLS IN THE *DROSOPHILA* OVARY

This chapter was published in Springer Methods [Laws and Drummond-Barbosa, Methods in Molecular Biology. 2015; 1328:57-72] and is reproduced here with minor edits. The purpose of the chapter is to provide a detailed methodological overview of FLP/FRT-mediated mosaic analysis in the Drosophila ovary, which is the major method used in this dissertation.

Introduction

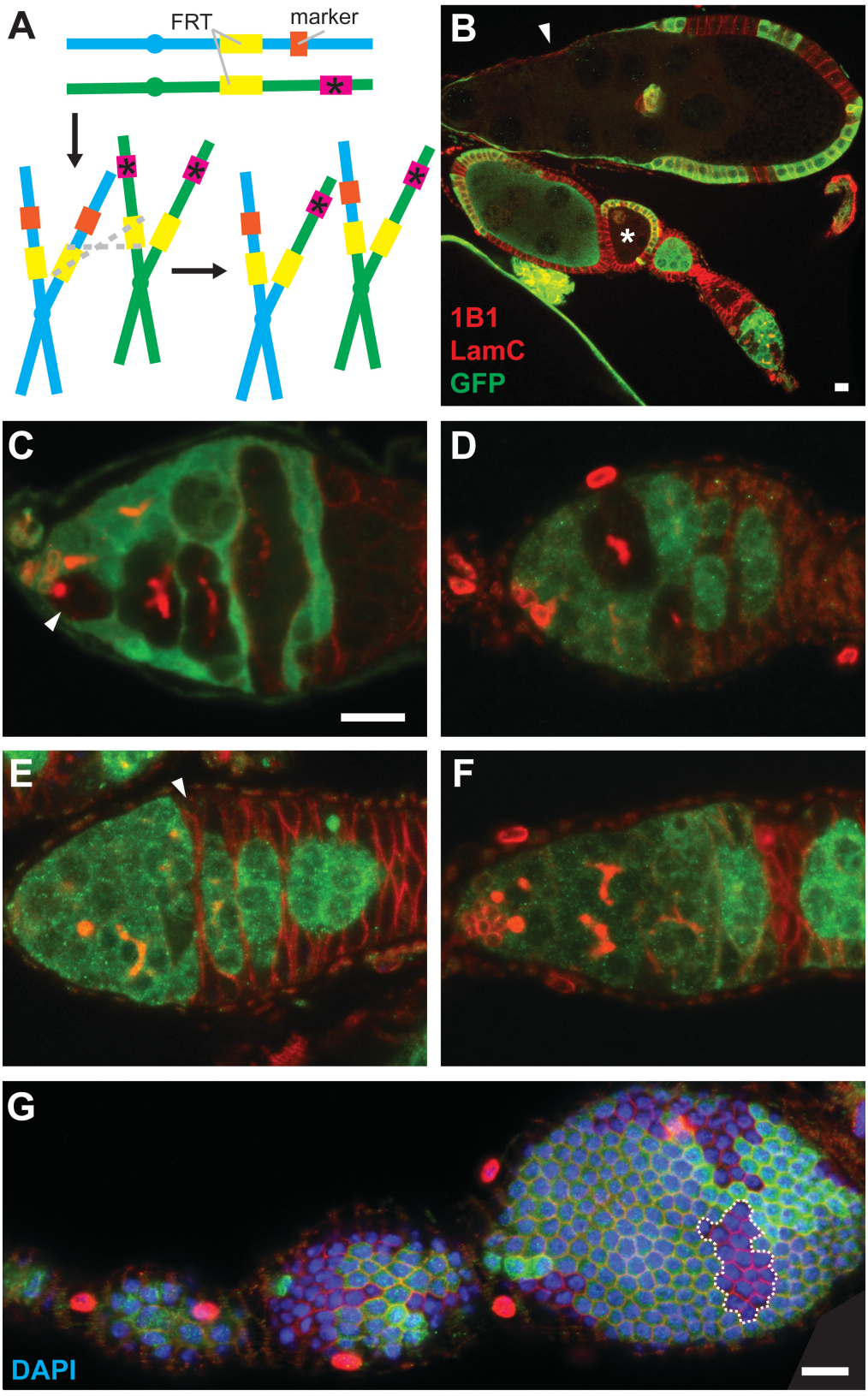
The ease of genetic mosaic generation in *Drosophila melanogaster* has allowed significant advances in understanding multiple aspects of stem cell biology and other processes during oogenesis (Perrimon 1998 , Theodosiou and Xu 1998). Genetic mosaic analyses, which typically involve the generation of identifiable, genetically distinct clones of cells within the context of wild-type tissue, allow the tracing of cell lineages, determining exact cells in which gene function is required, and distinguishing between cell autonomous and non-cell autonomous roles for genes. Genetic mosaics afford the added advantage of circumventing the lethality of mutations in essential genes, thereby uncovering their roles in later developmental stages.

Methods for the generation of mosaic animals have evolved over the years from technically challenging experimental manipulations involving transplantation, to the use of sophisticated genetic tools that facilitate mitotic recombination. In the classic quail-chicken chimera example, cells transplanted from quail embryos were distinguished from those of the host chicken embryo by the dense regions of heterochromatin in their nuclei, permitting the mapping of their fate during development (Le Lievre and Le Douarin 1975). In *Drosophila melanogaster*, transplantation of pole cells allowed the removal of gene function exclusively from the germline (Lehmann and Nusslein-Volhard 1987), and transplantation of imaginal

discs elucidated the tissue-autonomous and environmental factors influencing their developmental fate (Hadorn 1968). X-ray-induced mitotic recombination was useful in generating clones of mutant cells for the purpose of addressing cell autonomy of gene function (Korta et al. 2012). With the advent of molecular tools for inducible, site-specific mitotic recombination taking advantage of the yeast-derived flippase (FLP)/*FLP recognition target* (*FRT*) system (Chen et al. 2010), the use of genetic mosaic analysis in *Drosophila* has become commonplace.

Genetic mosaic analyses are very versatile. Typically, genetic mosaics are generated in the context of heterozygous organisms that carry *FRT* sequences at the base of specific chromosomes arms. One chromosome arm carries a mutation of interest, while its homolog has a wild-type allele of the corresponding gene and a readily identifiable marker, such as a ubiquitously expressed transgene encoding green fluorescent protein (GFP) or b-galactosidase (β-gal). In addition, a transgene encoding FLP under the control of a heat-shock inducible or tissue-specific promoter is present *in trans*. Once FLP expression is induced – for example, by heat-shocking the organism at a specific point during development or adulthood – cells can undergo FLP-mediated mitotic recombination through homologous *FRT* sequences, potentially generating unequal sister chromatids (Figure 2.1A). As sister chromatids segregate during mitosis, a homozygous mutant cell lacking the GFP (or b-gal) marker might be generated, forming a clone of GFP-negative mutant cells as it subsequently undergoes cell division rounds (Figure 2.1B-G). It should be noted, however, that numerous variations of this technique have been developed, involving the generation of positively marked mutant clones, clones for overexpression of transgenes or RNA hairpins for RNA

Figure 2.1. Confocal images of genetic mosaic ovarioles and germaria.
(A) GFP- or β-gal-negative mutant cells can be generated as unequal sister chromatids, produced as a result of FLP/*FRT*-mediated mitotic recombination (grey dashed lines), segregate during mitosis. Mutant allele is indicated by pink box and asterisk. Marker (orange box) is a constitutively expressed transgene encoding GFP or b-gal. **(B)** Mosaic ovariole containing previtellogenic (asterisk) and vitellogenic (arrowhead) follicles with GFP-negative germline cysts. **(C)** In a mosaic germarium, a GFP-negative GSC (arrowhead) gives rise to GFP-negative progeny. **(D)** A GSC loss event. GFP-negative germline cysts are present, but the original GFP-negative GSC is absent. **(E)** The FSC is located immediately anterior to the 2a/2b border, and it is recognizable as the anterior-most cell (arrowhead) in a GFP-negative follicle cell clone. (In region 2a, individual 16-cell cysts do not fill entire diameter of germarium, whereas in region 2B, lens-shaped 16-cell cysts span the breadth of germarium.) (**F**) When the FSC is lost, GFP-negative follicle cells can be detected, but the most anterior follicle cells are far posterior to 2a/2b. **(G)** A transient clone (dashed line) in a follicle cell monolayer provides an indirect readout for follicle cell proliferation. Absence of GFP (green) indicates marker-negative cells; 1B1 (red) labels fusomes and follicle cell membranes; Lamin C (LamC, red) labels cap cell nuclear envelopes; DAPI (blue) labels nuclei. Scale bars represent 10 μM. Images in **(C-F)** are shown at the same magnification.


A
FRT
marker
B
1B1
LamC
GFP
C
D
E
F
G
DAPI

interference, or wild-type clones for lineage tracing analysis (Lee and Luo 2001, Evans et al. 2009, Struhl and Basler 1993).

The focus of this chapter is how genetic mosaic analysis using adult-generated negatively-marked clones of cells in the germline or follicle cell lineage can be used to study a number of processes during *Drosophila* oogenesis that are known to be controlled by dietary conditions. Previous studies in our laboratory using this type of analysis have led to the identification of specific cells that require various nutrient-sensing or hormonal pathway components, allowing us to distinguish between direct versus indirect roles of systemic factors in controlling multiple distinct processes, including germline stem cell (GSC) and follicle stem cell (FSC) maintenance or proliferation, germline cyst growth and development, follicle cell proliferation, and vitellogenesis (Ables and Drummond-Barbosa 2010, Ables and Drummond-Barbosa 2013, Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, Hsu et al. 2008, LaFever and Drummond-Barbosa 2005, LaFever et al. 2010). The described protocol represents a detailed guide to strain generation, FLP/*FRT*-mediated clonal induction, ovary dissection and immunostaining, and data analysis.

Materials

Drosophila strains and culture conditions

1. Suitable *Drosophila* strains (see **Note 1**), including mutant stock of interest, heat-shock inducible flippase (*hs-Flp*) (see **Note 2**), *FRT* insertion on appropriate chromosome arm (see **Note 3**), and a corresponding *FRT* insertion recombined to a ubiquitously expressed marker, such as *ubi-GFP* or *arm-lacZ* (for GFP or β-gal expression, respectively).

2. G418 (Sigma) diluted in water to appropriate concentration, according to specific *FRT* insertion (see **Note 4**).

3. Standard fly culture media in a plugged vial.

4. Dry active yeast, such as used in baking.

5. Wet yeast paste: ~20 g active dry yeast thoroughly mixed into ~35 mL of dH_2O to the consistency of smooth peanut butter (see **Note 5**).

6. Water bath set at 37°C.

7. Vinyl-coated lead weight ring (or other weight of approximately 500 grams).

8. Kimwipes.

9. Plastic rack for fly vials.

10. Dissecting pin or thin spatula.

Dissection and immunostaining of ovaries

1. 1.5 mL microfuge tubes (*see* **Note 6**).

2. Glass or plexiglass dissection dish.

3. Kimwipes.

4. Glass Pasteur pipette and bulb.

5. Phosphate buffered saline (PBS).

6. Grace's Insect Medium (BioWhittaker).

7. 3% Bovine serum albumin (BSA; Sigma) prepared in water.

8. Washing solution: 0.1% Triton X-100 in PBS (see **Note 7**).

9. Blocking solution: 5% BSA, 0.1% Triton X-100, 5% normal goat serum in PBS (see **Note 8**).

10. Fixation solution: 5.3% formaldehyde in Grace's Insect Medium, prepared from 16% formaldehyde (Ted Pella) (see **Note 9**).

11. Primary antibodies: mouse anti-1B1 (Adducin-related protein; Developmental Studies Hybridoma Bank, DSHB), mouse anti-Lamin C (LC28.26; DSHB); chicken anti-GFP (Abcam) or chicken anti-β-gal (Abcam).

12. Secondary antibodies: anti-mouse Alexa Fluor 568 or 633 and anti-chicken Alexa Fluor 488 (Life Technologies).

13. Click-It Kit (Invitrogen), for EdU incorporation assay (see **Note 10**).

14. 4',6-diamidino-2-phenylindole (DAPI), for staining DNA.

15. Microscope slides and coverslips.

16. Weights of approximately 120 grams, for flattening mounted samples.

17. Stereomicroscope.

18. 2 pairs of sharpened forceps.

19. Tungsten needle and/or 27-gauge needle and syringe.

20. Nutator, for rotation of sample during fixation, washing, and immunostaining procedures.

Image acquisition and analysis

1. Confocal microscope, or equivalent microscopy set-up.

2. Image analysis software (such as ImageJ).

Methods

Overall, setting of the standard crosses to obtain control and experimental genotypes and performing the heat shock protocol described below take approximately two weeks if

starting from expanded, healthy fly stocks. Following the final heat-shock, the timing of dissection for clonal analysis is a crucial variable for the appropriate interpretation of results, as discussed in below.

***Drosophila* strains and culture conditions**

1. Generate a recombinant fly stock containing both the proximal *FRT* insertion and the mutant allele of interest on the same chromosome arm through standard crosses. *FRT* transgenes may carry different selection markers, but the majority include the neo^R marker (see **Notes 4** and **11**). To select for flies carrying the neo^R-containing *FRT* among progeny resulting from recombination cross (see **Note 12**), maintain the cross on food treated with G418 solution of appropriate concentration (see **Note 13**). Crosses should be transferred to fresh food every two days, such that the resulting progeny will be raised on G418 and thereby selected for the presence of the *FRT* insertion. Individual progeny should subsequently be screened for the presence of the mutant allele of interest for identification of flies carrying recombinant chromosome and balanced as a stock.

2. Generate flies of control and experimental genotypes (see **Note 14**) through standard crosses. At 0-2 days after eclosion (see **Note 15**), transfer females of appropriate genotypes along with sibling males to vials especially prepared for heat shock. These vials should include half of a folded Kimwipe directly covering the food surface to prevent the flies from sticking to it during heat shock.

3. Place flies in heat shock vials in a plastic rack, spreading vials out to allow easy water flow between them. Heat shock flies in the 37°C water bath, placing the weight on top of the vials to keep the rack underwater, and maintaining the appropriate water level to ensure that flies

are confined to the submerged portion of the vial. Heat shock should be conducted for one hour at a time, twice daily (see **Note 16**), for three consecutive days (see **Note 17**).

4. Following the final heat shock, transfer flies to vials supplemented with wet yeast paste, adding new males to the vials if some have died during heat shock. Transfer flies to vials containing fresh wet yeast daily until dissection. When selecting time points for dissection, consider the perdurance of both the marker used (see **Note 18**) and the protein of interest. Dissection time points up to ten days after heat shock will include both transient and permanent clones (Margolis and Spradling 1995) (see **Note 19**), which is an important consideration when interpreting the data. Multiple time points are typically included in the analyses.

Dissection and immunostaining of ovaries

1. Prepare Eppendorf tubes for dissected ovaries by filling them with 3% BSA solution (see **Note 20**).

2. Using a Pasteur pipette, transfer Grace's Insect Medium to a dissection dish (see **Note 21**).

3. Anesthetize flies using CO_2 and select females for dissection. Pick up females one at a time by gently pinching the thorax with sharp forceps.

4. Submerge each female in a dissection well filled with Grace's medium under a stereomicroscope. While holding females by the thorax, use the second pair of forceps to carefully pinch and pull away the posterior of the abdomen (at approximately two segments from the end). Ovaries should come out easily; otherwise, they can be pushed out of the abdomen.

5. Tease apart the anterior halves of ovarioles using a sharp tungsten needle or a fine-gauge needle in a syringe (see **Note 22**). Immobilize ovaries by holding on to their posterior end

using a pair of forceps and run the tungsten needle between ovarioles to tear the muscle sheath away from the anterior half.

6. Before transferring dissected ovaries to Eppendorf tubes, remove the BSA solution from Ependorf tubes using a Pasteur pipette, and discard the solution. This will also serve to coat the pipette with BSA and prevent ovaries from sticking to the glass. Use this coated pipette to transfer the dissected ovaries to the Eppendorf tube.

7. Repeat this process for all genotypes, minimizing the time between dissection and fixation. Ideally, the time between dissection and fixation should not exceed 30 minutes.

8. Fix ovaries in freshly prepared fixation solution for 13 minutes with rotation on a nutator at room temperature (see **Note 23**).

9. Rinse ovaries three times in washing solution by letting ovaries settle to bottom of the tube, then repeatedly changing the buffer. Wash four times for at least 15 minutes each on nutator at room temperature (see **Note 24**).

10. Block ovaries in blocking solution for at least three hours at room temperature or overnight at 4°C on nutator (see **Note 25**).

11. Stain ovaries with primary antibodies diluted in blocking solution: anti-1B1 (1:10), anti-Lamin C (1:100), and anti-GFP (1:2000). Primary antibody incubation times range from three hours at room temperature to overnight at 4°C on nutator.

12. Wash samples in washing solution four times for at least 15 minutes each on nutator (see **Note 26**).

13. Stain ovaries with secondary antibodies (anti-mouse Alexa 568 and anti-chicken Alexa 488) diluted 1:200 in blocking solution and protected from light with aluminum foil.

Secondary antibody incubation times range from one hour to five hours at room temperature on nutator.

14. Stain sample with 0.5 mg/mL DAPI in washing solution for 10 minutes at room temperature, protected from light, on nutator.

15. Wash sample in washing solution four times for at least 15 minutes each at room temperature, protected from light, on nutator.

16. Remove washing solution and add a small volume of the mounting medium of choice. (We use either Vectashield or 90% glycerol containing 20 mg/ml *n*-propyl gallate). Gently and thoroughly mix ovarioles with their mounting medium using a Pasteur pipette. Samples will keep at 4°C in the dark in mounting medium for extended periods of time (see **Note 27**).

17. To mount samples, transfer samples mixed with mounting medium onto a glass slide under a stereomicroscope. Using a pair of tungsten needles, carefully separate large late stage egg chambers from ovarioles and remove them from the slide. [For details on the staging of ovarian follicles, see Spradling (1993)]. The presence of large egg chambers on the slide will prevent the germaria from being sufficiently flattened by the mounting process, making it difficult to image them. Using tungsten needles, gently distribute ovarioles away from each other prior to adding the coverslip.

18. Add glass coverslip, cover it with a Kimwipe, and apply gentle pressure to the sample using a weight. This will flatten the ovarioles to facilitate imaging (see **Note 28**). Seal the coverslip using nail polish. Sealed, mounted slides will keep for extended periods of time at 4°C in the dark.

Image acquisition and analysis

Several general considerations in genetic mosaic analysis are crucial for accurate data interpretation. For example, perdurance of the protein of interest after removal of the cognate gene through mitotic recombination will depend on the stability of the protein and corresponding mRNA. Similarly, visualization of mutant cells will depend on the perdurance of GFP or β-gal markers. Finally, the marker expression level and the frequency of clone induction will vary depending on the specific marker and *FRT* insertions used for the experiments, respectively.

The types of images required vary depending on the type of analysis being conducted. We find it more efficient to acquire images for one type of analysis at a time rather than acquiring all types of images during the same microscopy sessions because the image acquisition mode may vary according to type of analysis. One should also be careful to avoid the analysis of damaged ovarioles [see Haack et al. (2013)] or those where immunostaining did not work well. The most common types of analyses performed in our lab are described below, starting with germline analyses involving ovarioles followed by those focused on the germarium, and ending with analyses of the follicle cell lineage.

Follicle growth and survival

1. The growth and survival of GFP- (or β-gal-) negative mutant germline cysts within developing follicles is assessed relative to flanking follicles containing GFP-positive cysts within the same ovariole (Figure 2.1B, asterisk; Figure 2.2A). In control mosaic ovarioles, GFP-negative follicles are larger than anterior and smaller than posterior flanking follicles. A deviation from this pattern in the experimental mosaics can reflect either a defect in cyst growth or premature death of the cyst. These two possibilities can be distinguished by co-staining ovaries with an apoptosis marker. Several dozen of ovarioles should be analyzed per

genotype at 10 days after the last heat shock. [For more precise quantification of the extent of cyst growth delay or overgrowth, see LaFever and Drummond-Barbosa (2005), LaFever et al. (2010)]

Progression through vitellogenesis

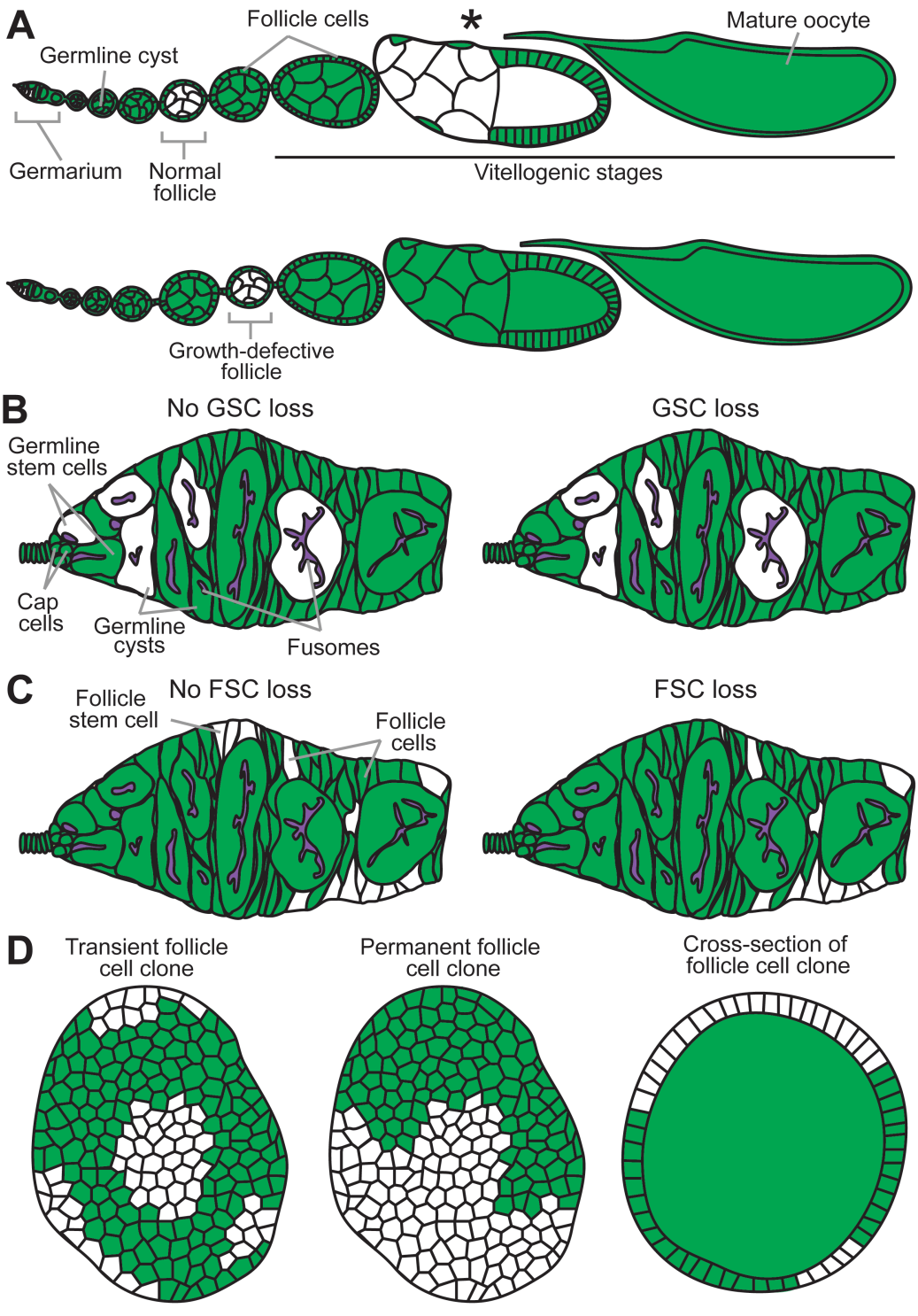
1. Vitellogenesis begins at stage 8 of oogenesis (Cummings et al. 1971). To assess the progression of mutant cysts through vitellogenesis, we quantify the fraction of ovarioles that contain a GFP-negative vitellogenic cyst in control versus mutant mosaic ovarioles (Figure 2.1B, arrowhead; Figure 2.2A, asterisk). Do not include any "artificially truncated" ovarioles (i.e. in which vitellogenic cysts have been inadvertently removed from the ovariole during dissection or mounting) in the analysis. Although degenerating vitellogenic egg chambers with pyknotic nuclei may also be directly detected in mosaic ovarioles, it is not possible to reliably score such egg chambers as GFP-negative or –positive. The ideal number of ovarioles scored per genotype will depend on the penetrance of the phenotype, but, at a minimum, several dozen should be analyzed at 10 days after heat shock.

2. An alternative method for quantifying vitellogenesis block involves exclusively analyzing mosaic ovarioles in which the entire germline is homozygous mutant, and scoring what percentage of ovarioles have vitellogenic versus dying follicles, in relation to equivalent control mosaics. Samples sizes, however, will be inevitably small, given the rarity of mosaic ovarioles containing a fully mutant germline.

GSC maintenance

1. Method one: measuring the occurrence of directly observable GSC loss events. In germaria where all transient clones have exited the germaria (Margolis and Spradling 1995), all GFP-negative cystoblasts and germline cysts will have arisen from a GFP-negative GSC

Figure 2.2. Diagrams of potential genetic mosaic analysis outcomes. (A) *Top*: Normal ovariole containing previtellogenic ("Normal follicle") and vitellogenic (asterisk) follicles with GFP-negative germline cysts. *Bottom*: Follicle containing GFP-negative cyst shows a delay in growth, readily apparent in comparison to neighboring wild-type follicles. **(B)** A permanent clone derived from an identifiable GFP-negative GSC (left) populates the germarium (*left*). A recent GSC loss event is recognizable by the presence of GFP-negative germline cystoblasts/cysts within a mosaic germarium without the original GFP-negative mother GSC (*right*). **(C)** Permanent clones arising from an identifiable GFP-negative FSC in the germarium (*left*) or without a GFP-negative FSC (right), which indicates a loss event. **(D)** Transient (*left*) and permanent (*middle*) follicle cell clones are imaged in single planes for quantification of follicle cell proliferation by clone size or EdU incorporation frequency, respectively. Cross-sections of follicle cell clones (*right*) in the ovariole are often visible during germline cyst analyses.


A
Germline cyst
Follicle cells
*
Mature oocyte
Germarium
Normal follicle
Vitellogenic stages
Growth-defective follicle
B
No GSC loss
GSC loss
Germline stem cells
Cap cells
Germline cysts
Fusomes
C
Follicle stem cell
No FSC loss
Follicle cells
FSC loss
D
Transient follicle cell clone
Permanent follicle cell clone
Cross-section of follicle cell clone

(Figure 2.1C, arrowhead; Figure 2.2B). To quantify GSC loss, we count the number of germaria that contain GFP-negative GSCs along with their GFP-negative progeny (Figure 2.1C and Figure 2.2B, left), versus similar germaria in which the original GFP-negative GSCs have been lost (i.e. the presence of GFP-negative germline cysts/cystoblasts in the absence of a GFP-negative GSC indicates that the GSC was lost from the niche) (Figure 2.1D and Figure 2.2B, right) (see **Note 29**). The number of germaria showing a GSC loss event as a fraction of all germaria containing mosaic germline can be directly compared among different control and experimental mosaics. This approach provides a snapshot of GSC loss events, and a single time point (e.g. 7-10 days after the last heat-shock) can be informative when comparing control and mutant mosaic germaria. A subtle GSC loss phenotype may not become apparent unless many germline mosaic germaria are analyzed, but approximately one hundred germaria per genotype represents a reasonable sample size.

2. Method two: calculating the fraction of ovarioles carrying GFP-negative GSCs over time. Quantify the number of germaria containing at least one GFP-negative GSC as a percentage of the total number of germaria in the sample (see **Note 30**). This proportion is sensitive to the recombination frequency of the *FRT*, so changes in the fraction of ovarioles containing GFP-negative GSCs should be tracked over time (e.g. 4-7 days, two weeks, three weeks, and four weeks after heat shock) in control and mutant mosaic germaria. Due to potential variability in the frequency of initial FLP/*FRT*-mediated recombination events, larger samples sizes (several hundred germaria per genotype per time point) allow more reliable measurements.

FSC maintenance

1. Currently, no reliable markers exist for FSCs, and they can only be unambiguously identified using a combination of criteria, including lineage tracing, morphology and position within germaria. Briefly, FSCs are the anterior-most somatic cells within follicle cell clones immediately anterior to the 2a/2b junction of the germarium (Figure 2.1E, arrowhead; Figure 2.2C, left). Follicle cells differ from more anteriorly located somatic cells, escort cells, by nuclear and cellular morphology (Sahai-Hernandez et al. 2012). The same general strategy described above to measure GSC loss can be used for FSCs (Figure 2.1E,F, and Figure 2.2C), with similar timing and sample size considerations.

Early cyst development

1. The number of early progeny of GFP-negative GSCs at different stages of development can be readily quantified in germaria containing at least one GFP-negative GSC. Germline cysts are staged by the morphology of their fusomes (de Cuevas and Spradling 1998) (Figure 2.1C; Figure 2.2B). After counting the numbers of GFP-negative cystoblasts, and 2-, 4-, 8-, and 16-cell cysts present within each germarium, those numbers are normalized to the number of GFP-negative GSCs within that same germarium. By comparing the average number of different early GFP-negative GSC progeny present in control versus mutant mosaic germaria, it is possible to detect changes in the relative frequencies of various stages, which can be the result of stage-specific delay, arrest or death of germline cysts. Alternatively, the relative distribution of early germline stages can be compared between GFP-negative versus GFP-positive GSC progeny within the same population of germaria of a given genotype, which has the advantage of minimizing any potential influence of genotype

background on the analyses. Analyzing several dozens of mosaic germaria per genotype at 7-10 days after heat shock should be sufficient to reveal differences in cyst distribution.

GSC proliferation

1. To directly measure the frequency of GSCs in S phase, we quantify the total number of mutant, GFP-negative GSCs that have incorporated the thymidine analog EdU as a percentage of all GFP-negative GSCs observed (see **Note 31**). This number can be compared to either incorporation of EdU in neighboring, marker-positive GSCs, or in marker-negative GSCs in control mosaics. Although this is a labor-intensive process, we recommend scoring several hundreds of GFP-negative GSCs per genotype for reliable results, unless differences in proliferation rates are enormous and readily apparent.

2. An indirect (and less labor intensive) readout of GSC proliferation is the number of progeny per GSC present in each germarium. Comparing the number of germline cysts per GFP-negative versus GFP-positive GSCs is a relative measure of the number of GSC divisions in the recent past, as long as problems with cystoblast/cyst survival are ruled out (see "Early cyst development" heading above).

FSC proliferation

1. As for GSCs, FSC proliferation can be detected by EdU incorporation. In this case, lineage analysis is used to identify FSCs as described above, and the number of EdU-positive FSCs as a fraction of all GFP-negative FSCs is compared between mutant and control mosaic germaria. As for GSC proliferation analysis, samples sizes should be large.

Follicle cell proliferation

1. The proliferation of follicle cells can also be directly measured by quantifying the number of EdU-positive follicle cells as a fraction of all GFP-negative follicle cells analyzed during mitotic stages of follicle development [egg chamber stages 2-6; see Spradling (1993)]. The percentage of EdU-positive follicle cells within the population of GFP-negative follicle cells can be compared to that of GFP-positive follicle cells within the same mutant mosaic ovarioles or to that of GFP-negative follicle cells in control mosaic ovarioles (Figure 2.2D). Dozens of ovarioles should be scored at 10 days after heat shock.

2. Alternatively, transient follicle cell clone size (e.g. 3 days after heat shock) quantification may serve as a readout for follicle cell proliferation during mitotically dividing stages [egg chamber stages 2-6; (Spradling 1993)]. GFP-negative clones should be compared in mutant and control mosaic ovarioles (Figure 2.1G, dashed outline; Figure 2.2D, *left*). One caveat of this approach, however, is that other factors (such as cell death or elimination) can also influence clone size. Dozens of clones should be analyzed per genotype.

Notes

1. Many of the necessary strains can be obtained from the Bloomington *Drosophila* Stock Center at Indiana University (flystocks.bio.indiana.edu).

2. Rather than employing *hs-Flp*, one could drive a *UAS-Flp* transgene in a spatially restricted pattern using a Gal4 line with specific expression pattern (Evans et al. 2009), although this eliminates temporal control.

3. *FRT* insertion should map to same chromosome arm as the mutation of interest, which should be distal to the *FRT*.

4. The concentration of G418 is calculated based on the active concentration of the drug and the level of resistance conferred by expression of the *neomycin resistance* (*neo*^{R}) transgene in different *FRT* insertion lines. G418 concentration should therefore be optimized for each specific *FRT* insertion, using appropriate positive and negative controls to ensure appropriate selection. For example, flies carrying one copy of the *FRT82B* insertion survive when raised on food treated with 30 mg/mL of active G418, while all control wild type flies die.

5. The consistency of yeast paste may change over time. We recommend storing prepared yeast paste at 4°C, covered with parafilm.

6. While 1.5 mL microtubes are usually used, smaller tubes may be used to conserve antibody, especially when ovary size is significantly reduced.

7. Immunostaining for the fusome marker 1B1 works best when Triton-X 100 is used, whereas for an alternate fusome marker, α-spectrin, we recommend Tween-20 instead.

8. The same detergent should be used in the washing and blocking solutions.

9. 16% FA keeps for one week at 4°C after being opened, after which fixation quality deteriorates. Fixation conditions must be optimized for each antibody, but antibodies described in this protocol work reproducibly well under these fixation conditions.

10. If using EdU incorporation kit, the Alexa Fluor 633 secondary antibody should be used instead of Alexa Fluor 568, which has a similar emission spectrum to the Click-It conjugate. The manufacturer's instructions should be used to visualize EdU.

11. Different *FRT* insertions vary in levels of *neo*^{R} expression, which is controlled by a heat-shock inducible promoter. While the leakiness of the promoter is often sufficient for selection on G418-treated fly food at room temperature, it is sometimes necessary to periodically heat-

shock flies at 37°C during the drug treatment for robust expression of *neo*^{R} (e.g. *FRT80B*) and effective selection.

12. The "recombination cross" is the cross between females carrying the *FRT* chromosome in trans to the mutation of interest and balancer males.

13. To prepare the fly food for G418 selection, etch a checkerboard pattern onto the surface of the pre-prepared food using a dissecting needle or thin spatula, then apply 200 mL of G418 solution. Dry food completely under a fume hood before transferring the crosses to the vials.

14. Experimental genotypes should carry the *FRT* insertion recombined to the mutant allele in *trans* to a corresponding wild-type *FRT* chromosome carrying a GFP or b-gal marker, in addition to the *hs-Flp* transgene on a separate chromosome. Control genotypes are virtually identical, with the exception that no mutant allele is present, such that marker-negative clones will be wild type.

15. To induce clones in the ovarian GSC niche, *Drosophila* should be heat shocked in the late larval and early pupal stages (Hsu and Drummond-Barbosa 2011) rather than in adult stages.

16. Heat shocks should ideally be eight to twelve hours apart.

17. Between heat shocks, transfer flies to regular fly food supplemented with dry yeast.

18. For example, we find that perdurance of GFP makes the identification of negatively marked GSCs difficult until four days after the last heat shock.

19. Transient clones are derived from mitotic recombination occurring within individual dividing progeny of the stem cells (which further divide to form clones) and, as oogenesis progresses, they disappear. In contrast, permanent clones are derived from a stem cell, and tend therefore to be much longer lasting than transient clones.

20. Reagents and freshly dissected ovaries can be kept at room temperature or on ice, depending on which particular cellular proteins or structures will be visualized by immunostaining. For example, if EdU incorporation assay will be performed, all reagents and dissected ovaries should be kept at room temperature.

21. Placing a black background under the dissecting dish helps with visualization of the ovaries during dissection and mounting.

22. For assays conducted on unfixed tissue (e.g. EdU incorporation), or for the visualization of intact terminal filament structures, do not tease ovarioles apart at this stage. In these cases, ovarioles can be teased apart following fixation by returning them to the dissection plate with wash buffer.

23. Optimal fixation and staining conditions depend on the antibody being used and should be established prior to conducting this analysis. These conditions work well for the antibodies noted in this protocol, which are routinely used in our laboratory.

24. Once fixation solution has been thoroughly rinsed from the sample, washes are very flexible. Depending on the antigen being detected, the sample can remain in wash solution for up to 2 weeks at 4°C.

25. Samples can remain in blocking solution for extended periods of time at 4°C.

26. After samples have been stained with primary antibody, they can be stored in washing solution for extended periods of time at 4°C.

27. Labile epitopes and the Click-it reaction used to detect EdU incorporation are exceptions and should be imaged as soon as possible.

28. The extent to which ovarioles should be flattened varies depending on the type of analysis to be conducted. For example, to obtain good single-plane images of the follicle

epithelium, additional weight (up to double) may be necessary. Conversely, samples lacking vitellogenic stages (such as those from flies on a poor diet) will be more easily flattened.

29. GSCs can be unambiguously identified by the presence of a stereotypically shaped, 1B1-positive fusome juxtaposed to the Lamin C-positive niche (Xie and Spradling 2000).

30. The percentage of ovarioles containing GFP-negative GSCs sometimes increase from early to later time points, possibly due to some GFP perdurance at early time points.

31. An increase in the percentage of EdU incorporation of GSCs could reflect either an increase in proliferation rates or a slower S phase. To distinguish between these possibilities, it is necessary to employ a secondary method of analysis (e.g., the use of a different cell cycle marker, such as the mitosis marker phosphorylated histone H3, or a direct comparison between the numbers of GSC progeny).

CHAPTER III

INSULIN-INDEPENDENT ROLE OF ADIPONECTIN RECEPTOR SINGALING IN *DROSOPHILA* GERMLINE STEM CELL MAINTENANCE

This chapter appeared in Developmental Biology [Laws, Sampson, and Drummond-Barbosa, Developmental Biology. March 15;399(2):226-36] and is reproduced here verbatim. Leesa LaFever Sampson generated the AdipoR^{27} allele and provided initial observations on the mutant. I performed all experiments included in the paper.

Introduction

Adipocytes comprise the largest endocrine organ in the body and actively contribute to energy homeostasis (reviewed in Kershaw and Flier 2004, Rosen and Spiegelman 2014). Not surprisingly, dysfunction of adipocytes as a result of obesity or lipodystrophy disrupts the function of other organs, increasing the risk of heart disease, stroke, diabetes, and cancers (Kizer et al. 2011, reviewed in Eckel et al. 2010, Trujillo and Scherer 2006, Vucenik and Stains 2012). Adipokines, which are adipocyte-secreted protein hormones, play a major role in mediating adipocyte effects on multiple tissues (reviewed in Cao 2014)). Leptin, for example, signals satiety to the brain and increases metabolism (reviewed in Bluher 2014), and mutations in leptin or its receptor cause obesity and metabolic defects in mice and humans (Clement et al. 1998, Zhao et al. 2014, Kakar et al. 2013, Zhang et al. 1994, Lee et al. 1996). Adiponectin has a well-known role in sensitizing peripheral tissues to insulin, and activation of adiponectin receptors is thought to increase insulin sensitivity through cell autonomous mechanisms (reviewed in Yamauchi and Kadowaki 2013). Adiponectin plasma levels are reduced in obese, insulin resistant, or diabetic individuals (reviewed in Yamauchi and Kadowaki 2013). Adiponectin-deficient mice are mildly glucose intolerant and insulin-

resistant (Kubota et al. 2002), and elevated serum expression of adiponectin is protective against diabetes in mice (Combs et al. 2004).

Adipocytes, together with hepatocyte-like oenocytes, constitute an endocrine organ termed the fat body in *Drosophila* (Liu et al. 2009), and recent studies have shown conservation of adipokine signaling modules in this organism (Rajan and Perrimon 2012, Kwak et al. 2013). For example, the cytokine Unpaired 2 (Upd2) is produced in adipocytes and acts on insulin-producing cells in the brain to control the secretion of insulin-like peptides (ILPs), and *upd2* mutant defects can be rescued by human leptin, despite a lack of primary sequence homology between the proteins (Rajan and Perrimon 2012). Similarly, *Drosophila* has no obvious adiponectin homolog, but AdipoR, the homolog of mammalian adiponectin receptors, was reported to act in insulin-producing cells to stimulate ILP secretion and control larval metabolism, and to mediate the effects of human adiponectin in *ex vivo* brain cultures (Kwak et al. 2013).

Despite the considerable focus on the insulin-sensitizing role of mammalian adiponectin in influencing metabolism, several lines of evidence suggest that adiponectin might also control stem cells. For instance, adiponectin induces the proliferation of muscle satellite cells (Fiaschi et al. 2009), hematopoietic stem cells, and adult hippocampal progenitors (DiMascio et al. 2007); increases endothelial progenitor numbers (Shibata et al. 2008); and stimulates regeneration of muscles and other tissues (Fiaschi et al. 2014). Conversely, adiponectin deficiency in mice leads to delayed liver regeneration, impaired recovery from renal damage, and delayed wound healing (Fiaschi et al. 2014). It remains unclear, however, whether the functions of adiponectin signaling in insulin sensitization and precursor/stem cell regulation are widely conserved, or whether the role of adiponectin in

stem cells relates to its effects on insulin sensitivity or is entirely distinct.

The *Drosophila* ovary is a powerful system for research on adult stem cell biology and its connection to whole-body physiology. The ovary is composed of ovarioles, which are strands of chronologically arrayed follicles (Figure 3.1A). Each follicle represents a germline cyst encapsulated by follicle cells, and is formed from stem cells in the anterior germarium (Figure 3.1B). Germline stem cells (GSCs) reside in a specialized niche, composed primarily of cap cells. GSCs are physically attached to cap cells via E-cadherin, and they also receive signals from the niche, including bone morphogenetic protein (BMP) ligands, which are required for maintenance of the stem cell fate (reviewed in Chen et al. 2011). Diet and insulin signaling also control GSCs and their differentiating progeny. On a rich diet, GSCs are well maintained, and GSCs and their progeny proliferate and grow faster than on a poor diet, with multiple diet-dependent factors mediating this response (Ables et al. 2012). For example, ILPs directly stimulate the germline to control GSC proliferation, cyst growth, and progression through vitellogenesis (LaFever and Drummond-Barbosa 2005, Hsu et al. 2008). In addition, ILPs act on niche cells to indirectly promote GSC maintenance (Hsu and Drummond-Barbosa 2009). No studies, however, have yet examined the role of adiponectin signaling in GSCs or any other *Drosophila* stem cell types.

In this study, we demonstrate a cell-autonomous, insulin-independent requirement for *AdipoR* in GSC maintenance in the *Drosophila* ovary through genetic mosaic analysis of a newly generated null *AdipoR* allele. *AdipoR* null GSCs are not fully responsive to BMP ligands and have a slight reduction of E-cadherin at the GSC-cap cell junction. Conversely, germline-specific overexpression of *AdipoR* inhibits natural GSC loss, suggesting that reduction in adiponectin signaling contributes to the normal decline in GSC numbers

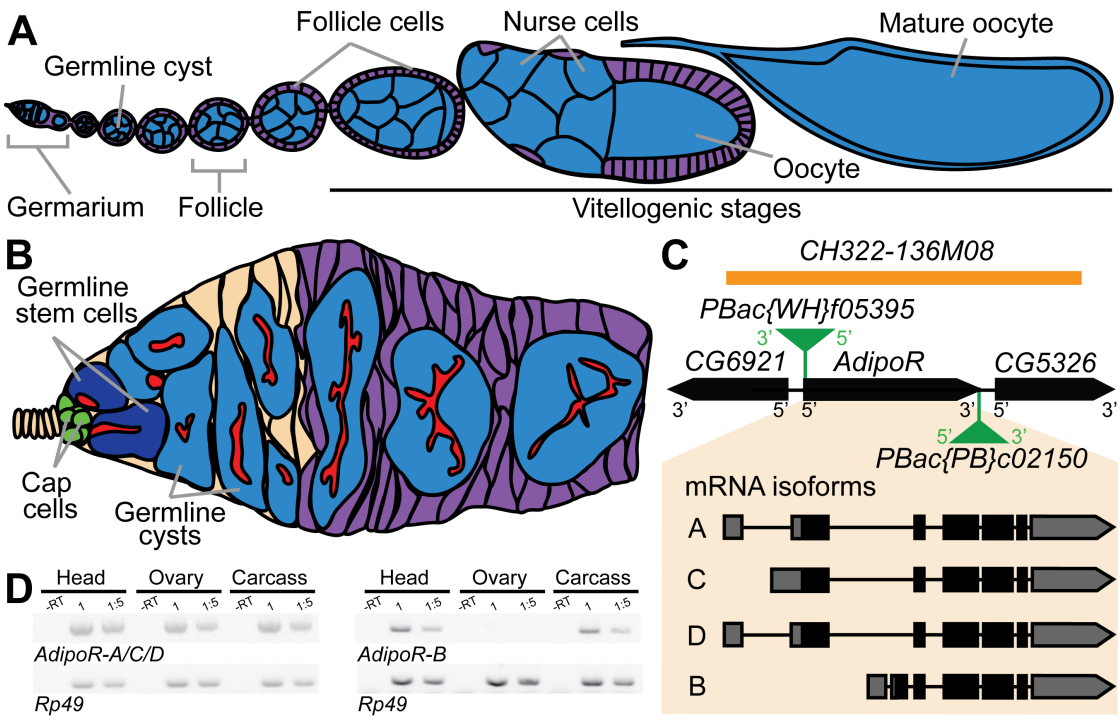


Figure 3.1 Generation of a null *AdipoR* allele for the study of its role in *Drosophila* oogenesis. (A) Each *Drosophila* ovariole is composed of a germarium followed by a strand of follicles containing a germline cyst (one oocyte and 15 nurse cells) surrounded by follicle cells. **(B)** The germarium houses a GSC population (dark blue) juxtaposed to somatic cap cells (green), a key component of the niche. GSCs divide asymmetrically to yield a GSC and a cystoblast, which forms 2-, 4-, 8-, and sixteen-cell germline cysts (light blue) through four rounds of cell division with incomplete cytokinesis. Sixteen-cell cysts are enveloped by follicle cells to form a follicle. GSCs contain an anteriorly anchored fusome (red). The fusome is a germline-specific organelle that adopts stereotypical conformations throughout GSC and germline cyst divisions, allowing the identification of each germ cell stage within the germarium (Hsu et al. 2008, de Cuevas and Spradling 1998). **(C)** The *AdipoR* gene encodes five mRNA isoforms corresponding to two distinct protein isoforms (AdipoR-A/C/D/E and AdipoR-B). For mRNA isoforms, boxes represent exons, with coding regions colored black. The genomic region is not to scale, and several potential non-coding RNAs have been omitted for clarity. The entire *AdipoR* gene was cleanly deleted by the recombination of flanking *PiggyBac* transposons (green triangles) to generate a null allele, $AdipoR^{27}$. The *VK37::AdipoR/CH322-136M08* genomic transgene (orange bar) was used for rescue experiments shown in Figure 3.5D and Table 3.2. **(D)** RT-PCR analysis of adult heads, ovaries, and carcasses showing differential expression patterns of *AdipoR-A/C/D/E* and *AdipoR-B*. *Rp49* is a control. PCR reactions were performed with undiluted or 1:5 dilutions of RT reactions. –RT, negative control with no reverse transcriptase in RT reaction.

observed over time in wild-type females. Surprisingly, we also found that *AdipoR* is not required for insulin sensitization of the germline, as GSC proliferation, cyst growth, and vitellogenesis remain unaffected upon loss of *AdipoR* function. Our findings establish *Drosophila* female GSCs as a new system for future studies addressing the molecular mechanisms whereby adiponectin receptor signaling modulates stem cell fate. Finally, we speculate that stem cell regulation might represent a distinct and evolutionarily more ancient function of adiponectin receptors relative to their role in insulin sensitization.

Materials and Methods

Drosophila strains and culture conditions

Fly stocks were maintained on standard cornmeal/molasses/yeast/agar medium at 22-25 °C. For experiments, females (in the presence of wild-type males) were transferred daily onto either standard medium supplemented with wet yeast paste ("rich diet") or molasses/agar ("poor diet") (Drummond-Barbosa and Spradling 2001). The *FRT82B AdipoR*^{27} recombinant chromosome and *VK37::AdipoR/CH322-136M08; AdipoR*^{27} fly lines were generated by standard crosses. The germline driver *PBac{GreenEye.nosGal4}Dmel2* (referred to here as *nos-Gal4::VP16*), *PBac[PB]c02150*, *PBac[WH]f05395*, and other genetic elements are described in FlyBase (http://www.flybase.org).

Generation of null *AdipoR*^{27} allele

Flies containing *PBac[PB]c02150* and *PBac[WH]f05395* were used to generate a flippase-mediated deletion of *AdipoR* (*AdipoR*^{27}), as described (Thibault et al. 2004). Primers used to verify recombination events and deletion using PCR are listed in Table 3.1.

Table 3.1. Primers used in AdipoR study.

Target	Forward	Reverse
AdipoR-B	DDB661[a] 5'- ACTAGTATCTCTGTTAGCCGCTC AAGATGCAGACCCAGCCGGAGGT-3'	DDB624[a] 5'- ACGCGTCTAAAAGGTTATTGGT TCGA-3'
CG5315^{c02150}	DDB203[b] 5'- TAAAACCCCTGCAATCGC-3'	DDB205[b] 5'- CCTCGATATACAGACCGATAA AC-3'
CG5315^{f05395}	DDB203[b] 5'- TAAAACCCCTGCAATCGC-3'	DDB201[b] 5'- CAACGTATGACCGCATTTCC-3'
AdipoR^{27}	DDB201[c] 5'-CAACGTATGACCGCATTTCC-3'	DDB203[c] 5'-TAAAACCCCTGCAATCGC-3'
AdipoR-A/C/D	DDB210[d] 5'- GATTCGGCCACTAATCTCCTC-3'	DDB 211[d] 5'- GATGCAGCCAAGAAGATGTG-3'
AdipoR-B	DDB 221[d] 5'-CCCAAAACCCTGACACGT-3'	DDB 211[d] 5'- GATGCAGCCAAGAAGATGTG-3'
Rp49	DDB137[d] 5'- CAGTCGGATCGATATGCTAAGC-3'	DDB138[d] 5'- AATCTCCTTGCGCTTCTTGG-3'

[a] Primers used for amplification of *LD23846* cDNA insert for generation of *pUASpI-AdipoR-B* transgene

[b] Primers used for detection of *FRT*-mediated recombination between *PBac{PB}c02150* and *PBac{WH}f05395* for generation of *AdipoR^{27}* allele

[c] Primers used to PCR across *AdipoR^{27}* and confirm *AdipoR* deletion

[d] Primers used for RT-PCR of indicated transcripts

Generation of *AdipoR* transgenic strains for rescue and overexpression analyses

The *AdipoR-A/C/D/E* cDNA was first subcloned from *LD23846* (*Drosophila* Genomics Resource Center, DGRC) into *pUASt* (Brand and Perrimon 1993) linearized with *EcoRI* and *XhoI*, and subsequently excised using *EcoRI* and *XbaI* prior to subcloning into *pUASpI* (Von Stetina et al. 2008) to generate *pUASpI- AdipoR-A/C/D/E*. The *AdipoR-B* coding region (including 20 bp immediately upstream of the initiation codon) was amplified from *IP14059* (DGRC) (see Table 3.1 for primers) and, after sequencing, subcloned into *pUASpI* using *SpeI* and *MluI* to generate *pUASpI-AdipoR-B*. Transgenic lines were generated as described (Rubin and Spradling 1983) either in house or by BestGene, Inc. (Chino Hills, CA).

For the *AdipoR* genomic rescue construct, BAC clone *CH322-136M08* (Figure 3.1C), obtained from BAC PAC Resources (https://bacpac.chori.org), was confirmed by sequencing and sent as a bacterial stab to Genetic Services (www.geneticservices.com) for integration into the *attP* docking site *VK37* on chromosome *2L* using FC31 integrase, as described (Venken et al. 2009). The *AdipoR* genomic insertion line is referred to as *VK37::AdipoR/CH322-136M08*, according to the suggested nomenclature (Venken et al. 2009).

Genetic mosaic and overexpression analyses

Females of genotype *hs-FLP/+; FRT82B AdipoR*/FRT82B Ubi-GFP* were generated by standard crosses. (*AdipoR** represents null $AdipoR^{27}$ or wild-type alleles of the *AdipoR* gene.) Zero- to 3-d-old females were maintained on dry yeast and heat shocked twice daily at 37 °C for 3 d to induce mitotic recombination (Xu and Rubin 1993). For GSC maintenance and cyst development and growth assays, flies were kept on a rich diet for 3 d after the final

heat shock, and then either maintained on a rich diet or shifted to a poor diet for 10 d prior to dissection and processing. *AdipoR*^{27} homozygous clones were identified by the absence of green fluorescent protein (GFP), as detected by antibody staining, and GSCs were identified based on their anterior location and typical fusome morphology (Hsu et al. 2008, de Cuevas and Spradling 1998). To quantify GSC loss, we analyzed all germaria that contained GFP-negative cystoblasts and/or cysts (derived from GFP-negative GSCs), and calculated the percentage of germaria that no longer contained GFP-negative GSCs (i.e. "GSC loss events"), as described (Hsu and Drummond-Barbosa 2009, Ables and Drummond-Barbosa 2010). Early germline cysts were staged according to their fusome morphology (de Cuevas and Spradling 1998), and later egg chambers were staged based on size, nuclear morphology, and yolk uptake (Spradling 1993). Follicle growth was qualitatively assessed by comparing follicles containing GFP-negative cysts to neighboring GFP-positive follicles. Progression through vitellogenesis was measured by determining the percentage of germline mosaic ovarioles containing a GFP-negative vitellogenic follicle (stage 8 or later) (Spradling 1993). Statistical significance was determined over at least two independent trials.

To measure GSC proliferation, flies were maintained on a rich diet for 10 d following the last heat shock, then either switched to a poor diet or maintained on a rich diet for an additional 3 d. EdU incorporation assays were performed as described (Ables and Drummond-Barbosa 2013). The number of EdU-positive GFP-negative GSCs was calculated as a percentage of the total number of GFP-negative GSCs analyzed over eight independent trials. Statistical significance was determined by Chi-square analysis and Student's *t* test.

For rescue experiments, *UAS-AdipoR-B* and *UAS-AdipoR-A/C/D/E* were individually recombined with *nos-Gal4::VP16*, and subsequently introduced into the *hs-FLP/+; FRT82B*

AdipoR^{27}*/FRT82B Ubi-GFP* genotype through standard crosses for genetic mosaic analyses. The *VK37::AdipoR/CH322-136M08* genomic transgene was similarly introduced into *hs-FLP/+; FRT82B AdipoR*^{27}*/FRT82B Ubi-GFP* females for genomic rescue experiment.

For overexpression analyses on a rich diet, *nos-Gal4::VP16*/*AdipoR-B* and *nos-Gal4::VP16/ AdipoR-A/C/D/E* females were raised at 18 °C to minimize transgene expression during development. Newly-eclosed females were maintained at 18 °C on a rich diet with wild-type males for 1 to 2 d, then switched to 29 °C for 10 or 20 d prior to dissections. For overexpression analyses on a poor diet, females were raised at 25 °C. Newly-eclosed females fed a rich diet with wild-type males for 1 to 2 d, then switched to a poor diet at 29 °C for 10 or 20 d prior to dissections.

Immunofluorescence and microscopy

Adult ovaries were dissected in Grace's Insect Medium (Lonza), teased apart, and fixed for 13 min in 5.3% formaldehyde (Ted Pella) in Grace's. Samples were rinsed and washed four times in 0.1% Triton X-100 (Sigma) in phosphate-buffer saline (PBS), or PBT, and blocked for at least 3 h at room temperature or overnight at 4 °C in 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (NGS; Jackson ImmunoResearch) in PBT unless otherwise noted. Samples were incubated at 4 °C overnight with primary antibodies in blocking solution at the following concentrations: mouse anti-Hts (1B1) (DSHB; 1:10); mouse anti-Orb (6H4) (DSHB; 1:10); mouse anti-Lamin C (LC28.26) (DSHB; 1:100); mouse anti-cleaved Caspase 3 (1:50, Cell Signaling); rabbit anti-Bruno (1:1000) (Sugimura and Lilly 2006); rabbit anti-GFP (1:2500, Torrey Pines); rabbit anti-Nanos (1:3000) (Hanyu-Nakamura et al. 2004); rabbit monoclonal anti-Smad3 (pMad; EP823Y) (1:100, Abcam); rat anti-E-cadherin (DCAD2) (1:3, DSHB); chicken anti-GFP (1:2000, Abcam); guinea pig anti-

A2BP1 (1:1000) (Tastan et al. 2010). For anti-A2BP1 labeling, ovaries were processed as described (Tastan et al. 2010). Ovaries stained with anti-pMad were dissected and fixed in Grace's medium supplemented with 25 mM Na_3VO_4 and 10 mM NaF. After primary antibody incubation, samples were washed for 2 h in PBT and incubated for 2 to 4 h in Alexa Fluor 488-, 568-, or 633-conjugated goat species-specific secondary antibodies (1:200, Invitrogen). Samples were mounted in Vectashield with DAPI (Vector Laboratories). Confocal images were acquired using a Zeiss LSM 700 microscope, and analyzed using either Zeiss ZEN 2009 or Axiovision software, and equally and minimally enhanced via histogram using Adobe Photoshop CS4. Quantification of pMad and E-cadherin levels was performed as described (Ables and Drummond-Barbosa 2013).

Apoptag Red In Situ Apoptosis Detection Kit (Millipore) was used following fixation and prior to antibody staining, as described (Drummond-Barbosa and Spradling 2001). EdU incorporation assays were performed as described (Ables and Drummond-Barbosa 2013). Briefly, ovaries were dissected in Grace's medium at room temperature, and incubated in 100 mM EdU (Invitrogen) in Grace's medium for 1 h prior to being teased apart, fixed, and stained as above. EdU was detected with AlexaFluor-594 via Click-It chemistry using manufacturer's instructions (Invitrogen) following secondary antibody incubation.

RT-PCR and qPCR analysis

For RT-PCR analysis, fifteen 0- to 3-d-old female *yw* flies were cultured overnight on standard medium supplemented with wet yeast paste. Head, ovaries, and carcasses of flies were dissected in RNA*later* (Ambion) for analysis of endogenous *AdipoR* isoforms. For qPCR analysis, 50 0-1 day old female *yw* flies were cultured for one week on either standard medium supplemented with wet yeast ("rich diet") or molasses/agar medium ("poor diet"),

then dissected in RNA*later*. In order to rule out the contribution of stage-specific differences in *AdipoR* expression, ovaries from flies raised on a rich diet were further processed to remove vitellogenic egg chambers, which are virtually absent in flies raised on a poor diet. RNA was extracted from all tissues using RNAqueous-4PCR DNA-free RNA Isolation for RT-PCR kit (Ambion) according to the manufacturer's instructions. cDNA was synthesized using SSRII kit (Ambion) according to manufacturer's instructions and used immediately for PCR using primers listed in Table 3.1. *Rp49* was used as a control for RT-PCR. For qPCR, reactions were performed with SYBER Green Supermix (Bio-Rad), and *Rp49*, *actin5C*, and *tubulin* were used as controls. Quantification of relative mRNA levels was performed using the comparative C_q method.

Results

Generation of the null *AdipoR*^{27} allele uncovers an essential role during development

Drosophila have no obvious adiponectin homolog based on primary sequence, but a single, well conserved AdipoR homolog is encoded (Figure 3.2). Five *AdipoR* mRNA isoforms are predicted, corresponding to two distinct protein isoforms, AdipoR-A/C/D/E and AdipoR-B (Figure 3.1C). Based on RT-PCR analysis, *AdipoR-A/C/D/E* and *AdipoR-B* mRNAs are both expressed in adult females; however, while *AdipoR-A/C/D/E* showed robust ovarian expression, *AdipoR-B* levels were below detection in the ovary (Figure 3.1D).

Functional analyses of the *AdipoR* gene in *Drosophila* have been limited by the lack of well-characterized genetic mutations in *AdipoR* (Kwak et al. 2013). Therefore, as a first step to analyze the role of *AdipoR* in the GSC lineage during *Drosophila* oogenesis, we generated a null allele of *AdipoR*, *AdipoR*^{27}, by *FLP/FRT*-mediated recombination between transposable elements flanking the *AdipoR* gene, and verified the molecular deletion (Figure 3.1C; Table

3.1). In the *AdipoR*^{27} allele, the transcribed region of *AdipoR* is completely removed, leaving neighboring genes intact. *AdipoR*^{27} homozygotes and *AdipoR*^{27}/*Df(3R)Excel6273*, *AdipoR*^{27}/*Df(3R)ED6090*, *AdipoR*^{27}/*Df(3R)ED6093*, or *AdipoR*^{27}/*Df(3R)ED6085* hemizygotes die during development, and a single copy of *VK37::AdipoR/CH322-136M08*, an *AdipoR* genomic rescue construct (Figure 3.1C), completely rescues this lethality (Table 3.2). Thus, *AdipoR* has an essential role during *Drosophila* development, precluding the analysis of ovaries from homozygous *AdipoR*^{27} females.

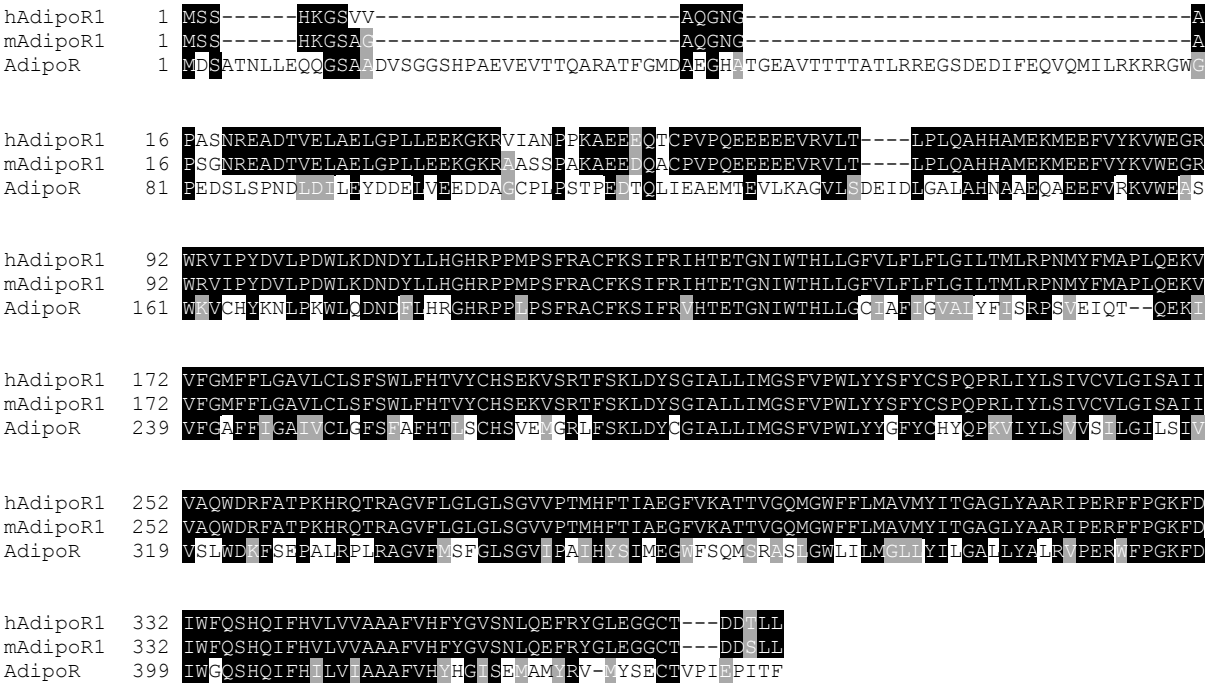
AdipoR is not required for GSC proliferation

Insulin signaling cell autonomously promotes GSC proliferation, germline cyst growth, and progression through vitellogenesis during *Drosophila* oogenesis (Drummond-Barbosa and Spradling 2001, LaFever and Drummond-Barbosa 2005, Hsu et al. 2008). To test if adiponectin receptor signaling sensitizes the germline to stimulation by ILPs, we generated genetic mosaic females and analyzed each of these insulin-dependent processes in homozygous null *AdipoR*^{27} GSC clones (recognized by the absence of a GFP marker) present in the context of neighboring wild-type cells.

To examine the requirement for *AdipoR* in GSC proliferation, we first measured the frequency of GFP-negative null *AdipoR*^{27} GSCs in S phase (based on incorporation of the thymidine analog EdU) compared to that of corresponding GFP-negative GSCs in control mosaic females in which all cells are wild-type (Figure 3.3A, B) on a rich diet, when high levels of circulating ILPs are available. Comparable frequencies of null *AdipoR*^{27} and control GSCs incorporated EdU (Figure 3.3C), indicating similar rates of proliferation. As an independent measure of GSC proliferation, we counted the number of progeny (i.e. cystoblasts and cysts) produced by null *AdipoR*^{27} GFP-negative versus control GFP-positive

Figure 3.2. Multiple sequence alignment between human, mouse, and *Drosophila* adiponectin receptor proteins. (A) *Drosophila* AdipoR is 52% identical to human AdipoR1 (hAdipoR1) and 52% identical to mouse AdipoR1 (mAdipoR1). **(B)** *Drosophila* AdipoR is 52% identical to hAdipoR2 and 53% identical to mAdipoR2 (www.ebi.ac.uk/Tools/msa/mafft). Black and grey indicate identical and similar amino acid residues, respectively (www.tcoffee.org).

A


hAdipoR1 1 MSS------HKGSVV----------AQGNG----------A
mAdipoR1 1 MSS------HKGSAG----------AQGNG----------A
AdipoR 1 MDSATNLLEQQGSAADVSGGSHPAEVEVTTQARATFGMDAEGHATGEAVTTTTATLRREGSDEDIFEQVQMILRKRRGWG
hAdipoR1 16 PASNREADTVELAELGPLLEEKGKRVIANFPKAEEDQTCPVPQEEEEEVRVLT----LPLQAHHAMEKMEEFVYKVWEGR
mAdipoR1 16 PSGNREADTVELAELGPLLEEKGKRAASSEAKAEEDQACPVPQEEEEEVRVLT----LPLQAHHAMEKMEEFVYKVWEGR
AdipoR 81 PEDSLSPNDLDILEYDDEIVEEDDAGCPLPSTPEDTQLIEAEMTEVLKAGVLSDEIDLGALAHNAAEQAEEFVRKVWEAS
hAdipoR1 92 WRVIPYDVLPDWLKDNDYLLHGHRPPMPSFRACFKSIFRIHTETGNIWTHLLGFVLFLFLGILTMLRPNMYFMAPLQEKV
mAdipoR1 92 WRVIPYDVLPDWLKDNDYLLHGHRPPMPSFRACFKSIFRIHTETGNIWTHLLGFVLFLFLGILTMLRPNMYFMAPLQEKV
AdipoR 161 WKVCHYKNLPKWLQDNDFLHRGHRPPLPSFRACFKSIFRVHTETGNIWTHLLGCIAFIGVAIYFISRPSVEIQT--QEKI
hAdipoR1 172 VFGMFFLGAVLCLSFSWLFHTVYCHSEKVSRTFSKLDYSGIALLIMGSFVPWLYYSFYCSPQPRLIYLSIVCVLGISAII
mAdipoR1 172 VFGMFFLGAVLCLSFSWLFHTVYCHSEKVSRTFSKLDYSGIALLIMGSFVPWLYYSFYCSPQPRLIYLSIVCVLGISAII
AdipoR 239 VFGAFFIGAIVCLGFSIAFHTLSCHSVEMGRLFSKLDYCGIALLIMGSFVPWLYYGFYCHYQPKVIYLSVVSILGILSIV
hAdipoR1 252 VAQWDRFATPKHRQTRAGVFLGLGLSGVVPTMHFTIAEGFVKATTVGQMGWFFLMAVMYITGAGLYAARIPERFFPGKFD
mAdipoR1 252 VAQWDRFATPKHRQTRAGVFLGLGLSGVVPTMHFTIAEGFVKATTVGQMGWFFLMAVMYITGAGLYAARIPERFFPGKFD
AdipoR 319 VSLWDRFSEPALRPLRAGVFMSFGLSGVIPAIHYSIMEGWFSQMSRASLGWLILMGLIYILGALLYALRVPERYFPGKFD
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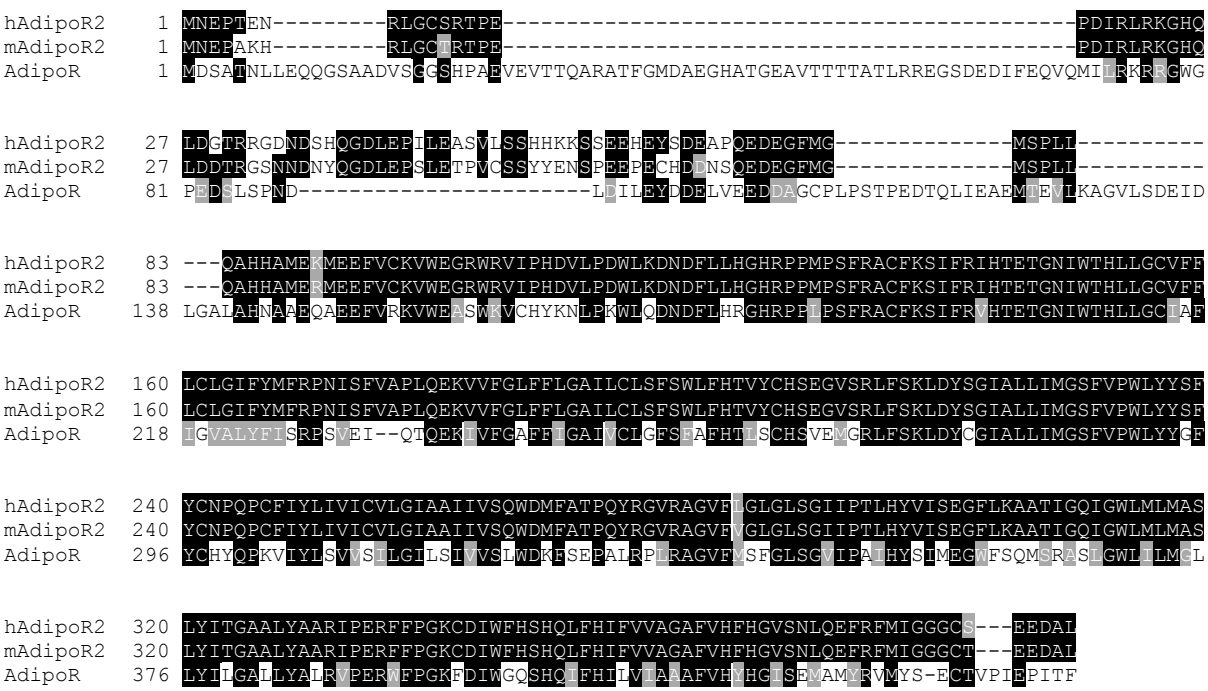
mAdipoR1 332 IWFQSHQIFHVLVVAAAFVHFYGVSNLQEFRYGLEGGCT---DDSLL
AdipoR 399 IWGQSHQIFHILVIAAAFVHYHGISEVAMYRV-MYSECTVPIEPITF

B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mAdipoR2 1 MNEPAKH---------RLGCTRTPE----------PDIRLRKGHQ
AdipoR 1 MDSATNLLEQQGSAADVSGGSHPAEVEVTTQARATFGMDAEGHATGEAVTTTTATLRREGSDEDIFEQVQMILRKRRGWG
hAdipoR2 27 LDGTRRGDNDSHQGDLEPILEASVLSSHHKKSSEEHEYSDEAPQEDEGFMG----------MSPLL----------
mAdipoR2 27 LDDTRGSNNDNYQGDLEPSLETPVCSSYYENSPEEPECHDLNSQEDEGFMG----------MSPLL----------
AdipoR 81 PEDSLSPND----------LDILEYDDEIVEEDDAGCPLPSTPEDTQLIEAEMTEVLKAGVLSDEID
hAdipoR2 83 ---QAHHAMEKMEEFVCKVWEGRWRVIPHDVLPDWLKDNDFLLHGHRPPMPSFRACFKSIFRIHTETGNIWTHLLGCVFF
mAdipoR2 83 ---QAHHAMERMEEFVCKVWEGRWRVIPHDVLPDWLKDNDFLLHGHRPPMPSFRACFKSIFRIHTETGNIWTHLLGCVFF
AdipoR 138 LGAIAHNAAEQAEEFVRKVWEASWKVCHYKNLPKWLQDNDFLHRGHRPPLPSFRACFKSIFRVHTETGNIWTHLLGCIAF
hAdipoR2 160 LCLGIFYMFRPNISFVAPLQEKVVFGLFFLGAILCLSFSWLFHTVYCHSEGVSRLFSKLDYSGIALLIMGSFVPWLYYSF
mAdipoR2 160 LCLGIFYMFRPNISFVAPLQEKVVFGLFFLGAILCLSFSWLFHTVYCHSEGVSRLFSKLDYSGIALLIMGSFVPWLYYSF
AdipoR 218 IGVALYFISRPSVEI--QTQEKIVFGAFFIGAIVCLGFSIAFHTLSCHSVEMGRLFSKLDYCGIALLIMGSFVPWLYYGF
hAdipoR2 240 YCNPQPCFIYLIVICVLGIAAIIVSQWDMFATPQYRGVRAGVFLGLGLSGIIPTLHYVISEGFLKAATIGQIGWLMLMAS
mAdipoR2 240 YCNPQPCFIYLIVICVLGIAAIIVSQWDMFATPQYRGVRAGVFVGLGLSGIIPTLHYVISEGFLKAATIGQIGWLMLMAS
AdipoR 296 YCHYQPKVIYLSVVSILGILSIVVSLWDKFSEPALRPLRAGVFMSFGLSGVIPAIHYSIMEGWFSQMSRASLGWLILMGL
hAdipoR2 320 LYITGAALYAARIPERFFPGKCDIWFHSHQLFHIFVVAGAFVHFHGVSNLQEFRFMIGGGCS---EEDAL
mAdipoR2 320 LYITGAALYAARIPERFFPGKCDIWFHSHQLFHIFVVAGAFVHFHGVSNLQEFRFMIGGGCT---EEDAL
AdipoR 376 LYILGALLYALRVPERYFPGKFDIWGQSHQIFHILVIAAAFVHYHGISEVAMYRVMYS-ECTVPIEPITF

GSCs within *AdipoR*^{27} mosaic germaria on a rich diet, and found that those numbers were equivalent (Figure 3.3D). These results indicate that *AdipoR* is not necessary for ILPs to promote GSC proliferation on a rich diet.

We reasoned that *AdipoR* might be required when nutrients are limiting to enable GSCs to respond effectively to low levels of ILPs. As expected, the fraction of GFP-negative GSCs in S phase was reduced in control mosaic females on a poor diet compared to those on a rich diet (Figure 3.3C), in accordance with the known effects of diet on GSC proliferation (Drummond-Barbosa and Spradling 2001, Hsu et al. 2008). The frequency of EdU incorporation in *AdipoR*^{27} GSCs, however, was statistically indistinguishable from that in control GSCs on a poor diet (Figure 3.3C). Correspondingly, null *AdipoR*^{27} GFP-negative and control GFP-positive GSCs in *AdipoR*^{27} mosaic germaria yield comparable numbers of progeny on a poor diet (Figure 3.3D). We therefore conclude that *AdipoR* is not required for the sensitization of GSCs to either high or low levels of ILPs during their proliferative response to diet.

AdipoR is not required for cyst growth or vitellogenesis

Although GSCs do not require *AdipoR* for their proliferation, stem cells and their progeny can differ in their requirements during the ovarian response to diet (LaFever et al. 2010). Therefore, we directly tested whether *AdipoR* might modulate ILP-sensitivity of the differentiating progeny of GSCs during cyst division, growth, or progression through vitellogenesis. GFP-negative *AdipoR*^{27} cystoblasts, and two-, four-, eight-, and 16-cell cysts were as equally well represented as GFP-positive control cystoblasts and cysts in the same germaria, or as GFP-negative wild-type cystoblasts and cysts in control mosaic germaria on

Table 3.2. *CH322-136M08* fully recues *AdipoR*^{27} lethality

Genotype[a]	*AdipoR* status	*CH322-136M08* present?	Number of adults (Percentage of total)
+/*CyO*; *Df(3R)Exel6274* or *AdipoR*^{27}/*TM3*	Heterozygous	No	22 (24.4%)
AdipoR/CH322-136M08*/+; *Df(3R)Exel6274* or *AdipoR*^{27}/*TM3	Heterozygous	Yes	44 (48.9%)
+/*CyO*; AdipoR^{27}/Df(3R)Exel6274	Homozygous	No	0 (0%)
AdipoR/CH322-136M08*/+; *AdipoR*^{27}/*Df(3R)Exel6274	Homozygous	Yes	24 (26.7%)

[a]Total number of adult progeny of specified genotypes obtained from *Df(3R)Exel6274*/TM3 x *VK37::AdipoR/CH322-136M08*/CyO; *AdipoR*^{27}/TM3 cross from eclosion of initial progeny over five days.

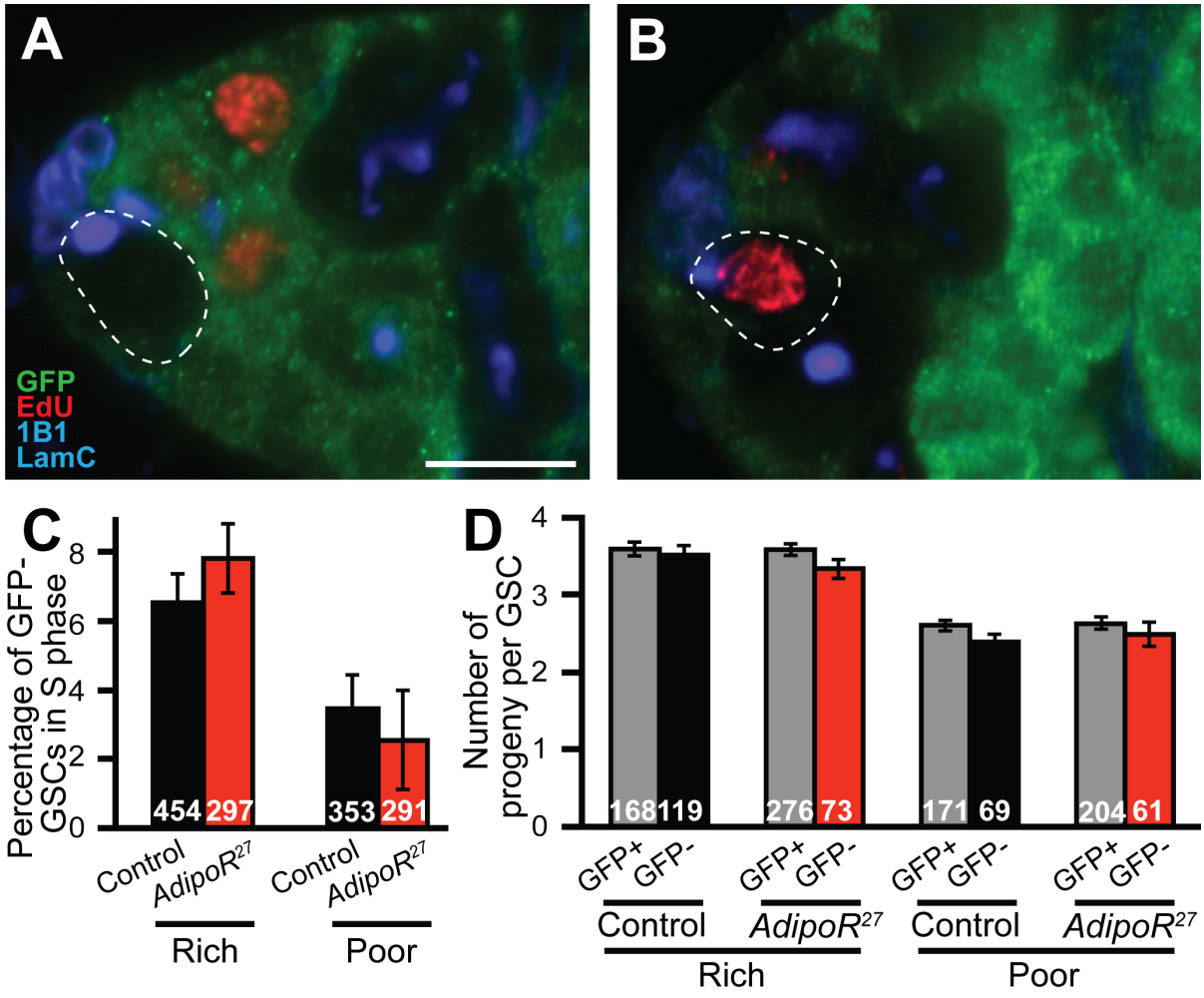


Figure 3.3. *AdipoR* is not required cell autonomously for GSC proliferation. (A and B) *AdipoR*^{27} mosaic germaria containing GFP-negative GSCs (dashed lines) without (A) or with (B) EdU incorporation (red). GFP (green) labels wild-type cells; Lamin C (LamC; blue) labels cap cell nuclear envelopes; 1B1 (blue) labels fusomes. Scale bar, 10 μm. **(C)** Average percentage of GFP-negative GSCs that incorporate EdU in control and *AdipoR*^{27} mosaic germaria. Total number of GFP-negative GSCs analyzed is included inside bars. **(D)** Average number of cystoblasts and germline cysts per GFP-positive or GFP-negative GSC in control and *AdipoR*^{27} mosaic germaria. Total number of GSCs analyzed is included inside bars. See Figure 3.4A for distribution of cystoblast/cyst stages. Error bars represent S.E.M. Differences are not significant by Chi-Square analysis or Student's *t* test.

either rich or poor diets (Figure 3.4A). These results indicate that early germline divisions were unperturbed by loss of *AdipoR*. Likewise, *AdipoR* was not required for cyst growth because GFP-negative germline cysts within follicles in $AdipoR^{27}$ mosaic ovarioles developed at normal rates relative to neighboring GFP-positive cysts and to control GFP-negative cysts regardless of diet (Figure 3.4B; 40 GFP-negative $AdipoR^{27}$ cysts and 32 GFP-negative control cysts analyzed). To determine if *AdipoR* is required for vitellogenesis, we measured the frequency of ovarioles containing GFP-negative cysts within vitellogenic follicles in $AdipoR^{27}$ compared to control mosaic females (Figure 3.4C, D). Over one-third of $AdipoR^{27}$ mosaic ovarioles contained at least one GFP-negative vitellogenic germline cyst, comparable to what we observed in control mosaics (Figure 3.4D). Together with the GSC proliferation results, these data indicate that *AdipoR* is not required for insulin-dependent processes in the ovarian germline, suggesting that adiponectin signaling does not have a role in insulin sensitization in this context.

AdipoR is cell autonomously required for GSC maintenance

Adiponectin has reported roles in mammalian progenitor cells (reviewed in Fiaschi et al. 2014). It remains unclear, however, whether the control of stem cells by adiponectin signaling is linked to the effect of adiponectin on insulin sensitization. *AdipoR* is clearly not required for GSC proliferation (Figure 3.3), an insulin-dependent process (LaFever and Drummond-Barbosa 2005). We therefore tested if *AdipoR* is cell autonomously required for GSC maintenance, which is a diet-dependent process but does not require insulin signaling within the germline (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, Yang et al. 2013). We measured the occurrence of GSC loss events in control and $AdipoR^{27}$

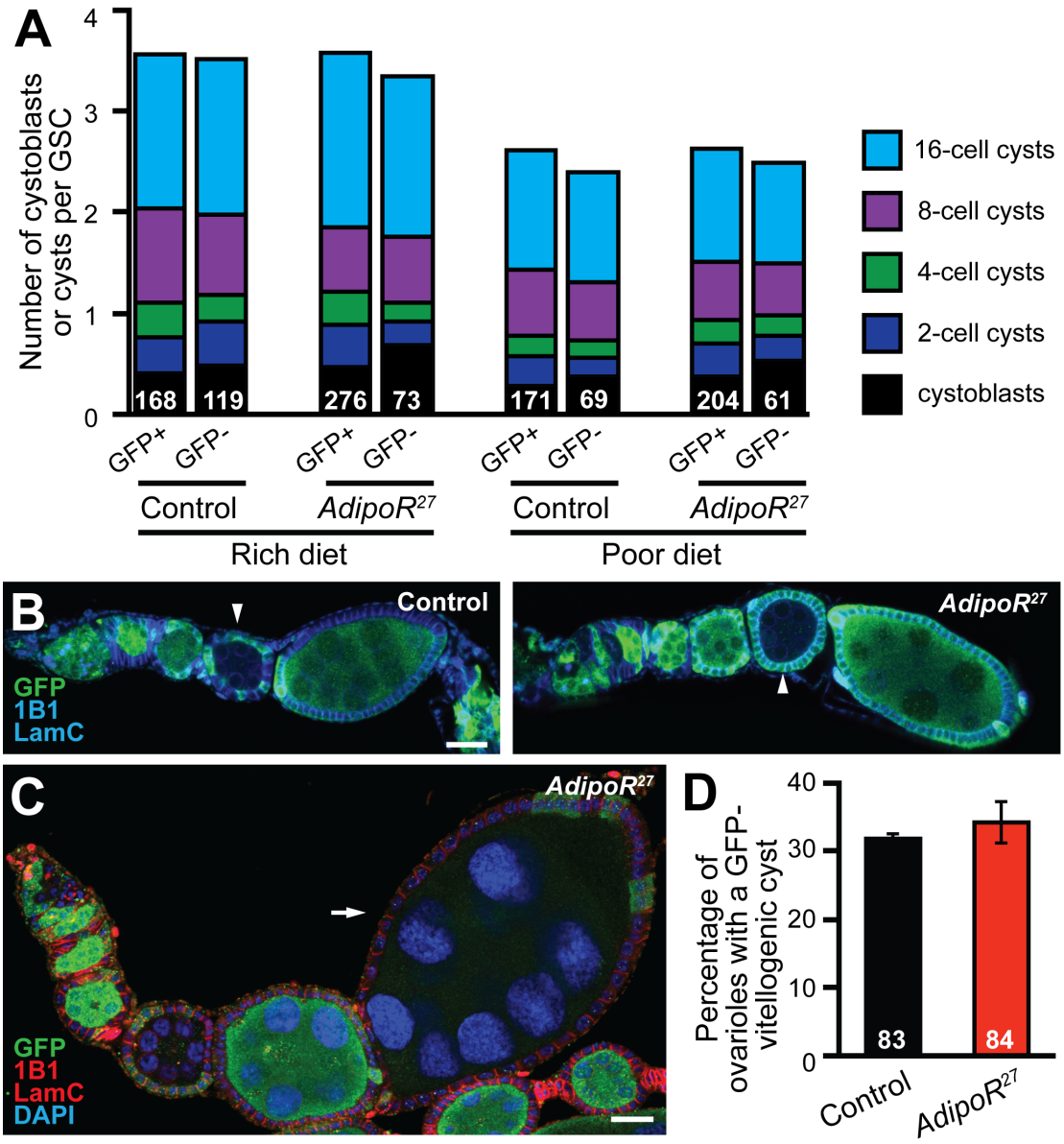


Figure 3.4 *AdipoR* is not cell autonomously required for cyst division, growth, or progression through vitellogenesis. (A) Average distribution of cystoblasts, 2-, 4-, 8-, and sixteen-cell cysts per GSC in control and *AdipoR*^{27} mosaic germaria maintained on rich or poor diets. The same data are plotted in Figure 3.3D. **(B)** Control (left) and *AdipoR*^{27} (right) mosaic ovarioles displaying GFP-negative germline cysts (arrowheads) that grow normally relative to flanking GFP-positive cysts. GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes and follicle cell membranes; LamC (blue) labels cap cell nuclear envelopes. **(C)** *AdipoR*^{27} mosaic ovariole showing a homozygous mutant GFP-negative germline cyst (arrow) in vitellogenesis. GFP (green) labels wild-type cells; 1B1 (red) labels fusomes and follicle cell membranes; LamC (red) labels cap cell nuclear envelopes; DAPI (blue) labels nuclei. Scale bars, 20 μm. **(D)** Percentage of control and *AdipoR*^{27} mosaic ovarioles with vitellogenic follicles containing GFP-negative germline cysts. Total numbers of ovarioles analyzed are included inside bars. Error bars represent S.E.M. Differences are not significant by Chi-Square analysis or Student's *t* test.

mosaic germaria on both rich and poor diets (Figure 3.5A-C). Wild-type GFP-negative GSCs in control mosaics are lost in less than 5% of germaria containing germline clones on a rich diet, and the percentage of control mosaic germaria showing GSC loss increases to 18% on a poor diet, as expected (Figure 3.5C) (Hsu and Drummond-Barbosa 2009). We found that null *AdipoR*^{27} GSCs are lost at significantly higher rates than control GSCs on both diets (Figure 3.5C), and these loss rates are comparable to those of previously described GSCs defective for maintenance genes (LaFever et al. 2010, Ables and Drummond-Barbosa 2010). GSC loss is fully rescued by *VK37::AdipoR/CH322-136M08*, a genomic *AdipoR* rescue transgene (Figure 3.1C, 3.5D). These results indicate an intrinsic requirement for *AdipoR* in GSC maintenance.

The fold increase in *AdipoR*^{27} GSC loss relative to controls is diet dependent. *AdipoR*^{27} GSCs are lost five times more frequently than control GSCs on a rich diet, whereas *AdipoR*^{27} GSC loss is only two-fold higher than for controls on a poor diet (Figure 3.5C). These results imply that a partial reduction in AdipoR signaling contributes to the increase in wild-type GSC loss observed on a poor diet. *AdipoR* mRNA levels do not differ dramatically in response to diet (Figure 3.6), suggesting that regulation of AdipoR signaling might occur at the level of either AdipoR protein or other upstream or downstream pathway components.

AdipoR is predicted to encode two distinct protein isoforms, AdipoR-A/C/D/E and AdipoR-B (Figure 3.1C). To determine whether a specific isoform promotes GSC maintenance, we tested the ability of germline-expressed transgenes encoding each of the isoforms to rescue the *AdipoR*^{27} GSC loss observed in mosaic females. We generated *AdipoR*^{27} genetic mosaic germaria in females expressing *UAS-AdipoR-A/C/D/E* or *UAS-AdipoR-B* transgenes under the control of the germline-specific *nos-Gal4::VP16* driver.

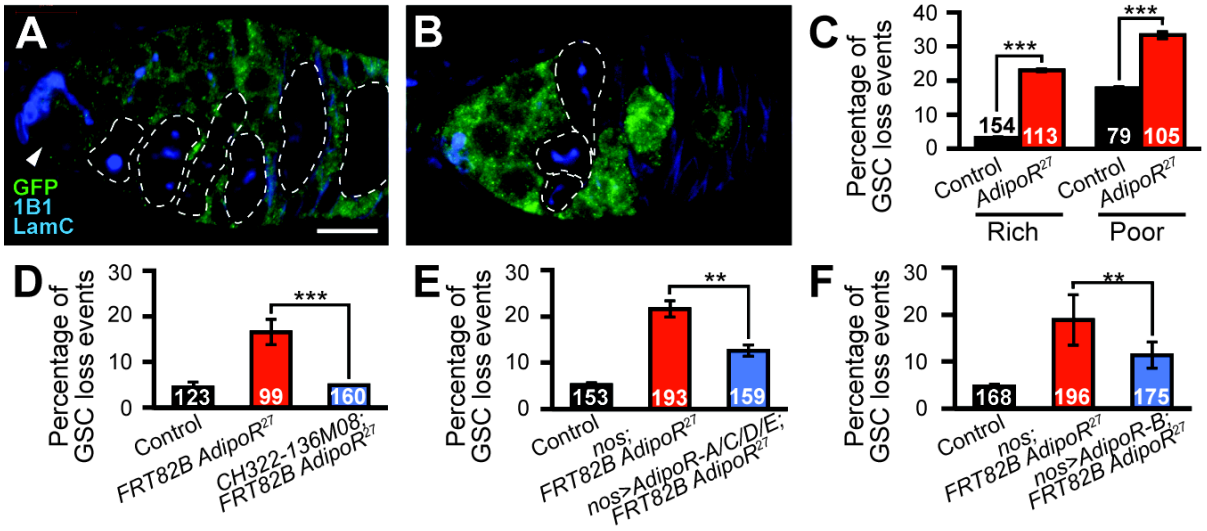


Figure 3.5. ***AdipoR* is required cell autonomously for GSC maintenance. (A and B)** Genetic mosaic germaria showing GFP-negative germline cystoblasts and cysts (outlined) clonally arisen from GFP-negative GSCs (arrowhead) (A). The presence of GFP-negative germline cysts without a GFP-negative GSC indicates a GSC loss event (B). GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes; LamC (blue) labels cap cell nuclear envelopes. Scale bar, 10 μm. **(C)** Quantification of GSC loss events in control and *AdipoR*^{27} mosaic germaria on rich and poor diets showing significant loss of *AdipoR*^{27} GSCs at 13 days after clone induction. **(D-F)** Graphs showing rescue of *AdipoR*^{27} GSC loss by *VK37::AdipoR/CH322-136M08*, a genomic rescue construct spanning the *AdipoR* gene (see Figure 3.1C) **(D)**, or by germline-driven expression of *UAS* transgenes encoding *AdipoR-A/C/D/E* **(E)** or *AdipoR-B* **(F)**. Total numbers of germaria with mosaic germline counted are included above or inside bars. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, Chi-Square analysis. Error bars represent S.E.M.

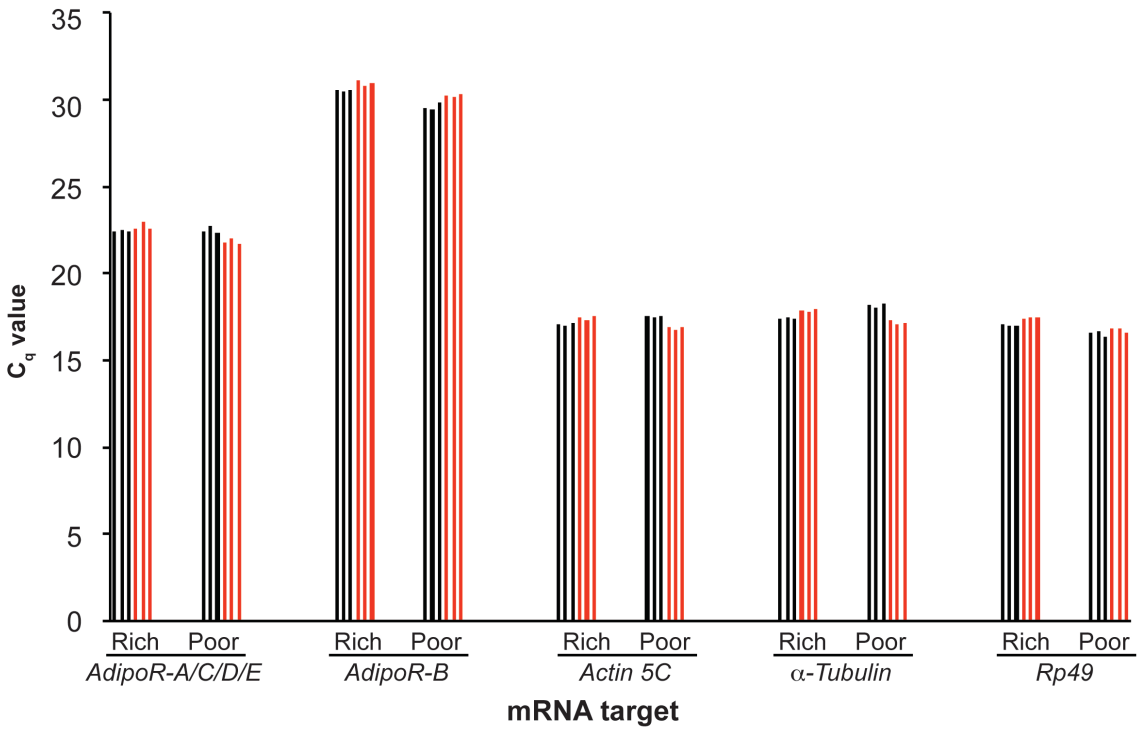


Figure 3.6. mRNA levels of *AdipoR* isoforms do not appear to be diet-dependent. C_q values from qPCR targeting *AdipoR-A/C/D/E* and *AdipoR-B* transcripts from the ovaries of females raised either a rich or a poor diet are plotted. Black and red bars represent independent biological replicates, with individual bars representing technical replicates within a single experiment.

Germline expression of either *AdipoR-A/C/D/E* or *AdipoR-B* partially restores *AdipoR*^{27} GSC maintenance (Figure 3.5E, F), and the partial rescue likely reflects relative low levels of transgene expression.

AdipoR null GSCs are not lost through apoptosis

We next tested if *AdipoR* promotes GSC maintenance by preventing apoptosis. We examined GFP-negative GSCs in *AdipoR*^{27} and control mosaic germaria for expression of cleaved Caspase 3, an early apoptosis marker, and found no evidence for GSC apoptosis (Figure 3.7A, B). Similar results were obtained using ApopTag, a marker of late apoptosis (Figure 3.7C, D). Both cleaved Caspase 3 and ApopTag labeled a fraction of later germ cells within both *AdipoR*^{27} and control mosaic germaria (Figure 3.7), ruling out technical difficulties with these apoptosis markers. These data suggest that *AdipoR* mutant GSCs are not lost through apoptosis, although we cannot completely rule out alternative cell death mechanisms.

AdipoR is required for robust levels of E-cadherin at the niche-GSC junction and full response of GSCs to BMP ligands

GSCs physically reside in a niche environment, and both the size of that niche and the competence of GSCs to adhere to it contribute to their maintenance (Song et al. 2002). We therefore tested whether *AdipoR* in GSCs controls the size of the niche and GSC adhesion to cap cells. *AdipoR*^{27} mosaic germaria with either one or two mutant, GFP-negative GSCs do not have significantly fewer cap cells than those with no mutant GSCs (Figure 3.8A,B). This indicates that AdipoR signaling in GSCs does not have a non-cell autonomous role in controlling insulin signaling in the niche. Additionally, we measured E-cadherin levels at the

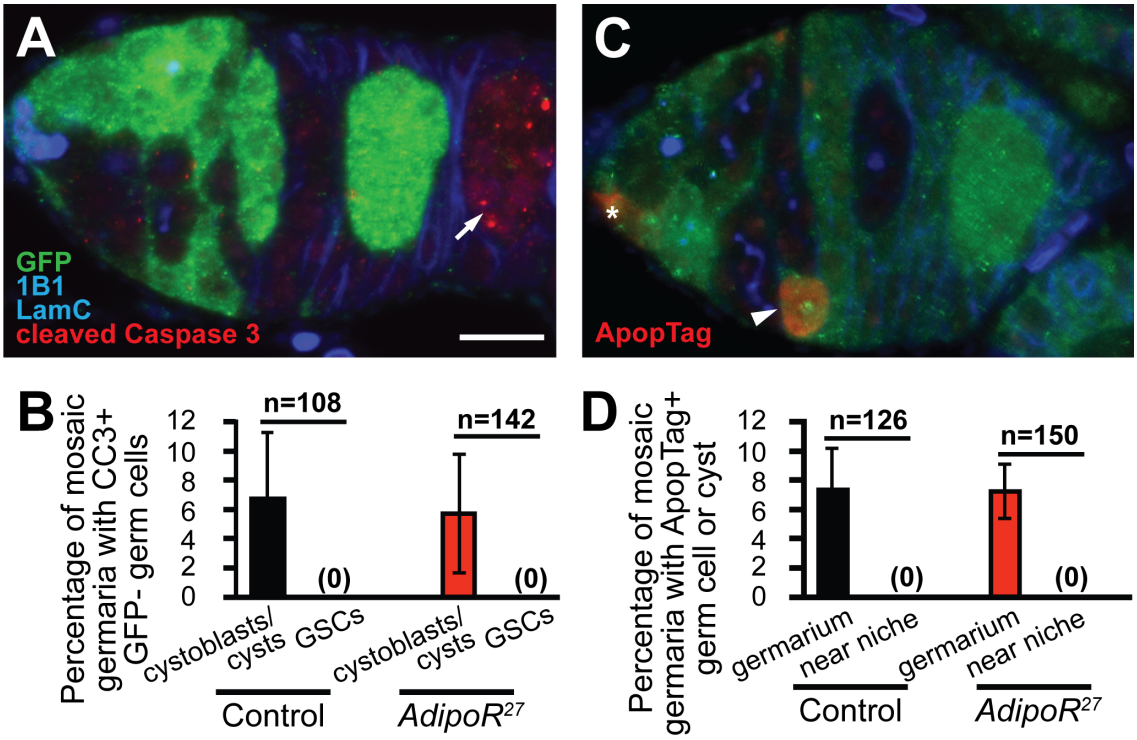
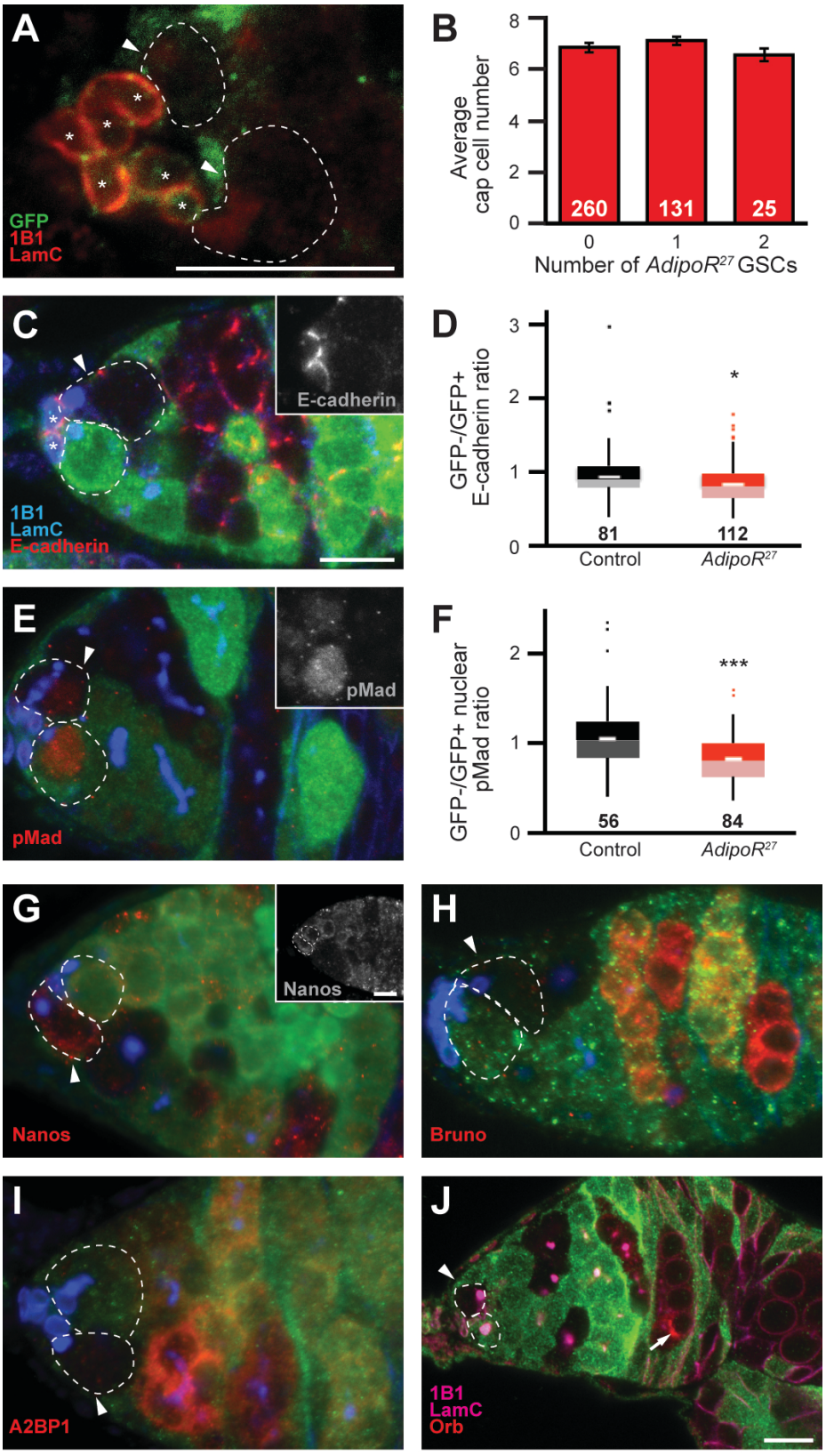


Figure 3.7. ***AdipoR*^{27} GSCs are not lost through apoptosis. (A)** *AdipoR*^{27} mosaic germarium containing a GFP-negative, cleaved Caspase 3 (red)-positive germline cyst (arrow). **(B)** Percentage of germaria with cleaved Caspase 3-positive, GFP-negative cystoblasts/cysts or GSCs. Total numbers of germaria analyzed are included above. **(C)** *AdipoR*^{27} mosaic germarium containing a GFP-negative, ApopTag (red)-positive germline cyst (arrowhead) away from the niche. Asterisk indicates an ApopTag-positive somatic cell. GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes; LamC (blue) labels cap cell nuclear envelopes. Scale bar, 10 μm. **(D)** Quantification of control and *AdipoR*^{27} mosaic germaria containing ApopTag-positive germ cells. GFP status or specific stage of ApopTag-positive germ cells cannot be reliably scored at this late stage of apoptosis. Total numbers of germline mosaic germaria counted are included above. Differences are not statistically significant by Chi-Square analysis. Error bars represent S.E.M.

Figure 3.8. *AdipoR* is required for full levels of BMP signaling and E-cadherin at the GSC-cap cell junction, but not for control of known differentiation markers. (A) *AdipoR*^{27} null GSCs (GFP-negative) have a subtle, but significant, decrease in E-cadherin (red; greyscale image shown in inset) levels at the junction of GSCs and cap cells (asterisks) compared to neighboring GFP-positive control GSCs. **(B)** Box-and-whiskers plot showing the ratio of E-cadherin levels at the GSC-cap cell junction for GFP-negative to those for neighboring GFP-positive GSCs in control and *AdipoR*^{27} mosaic germaria. **(C)** Nuclear phosphorylated Mad (pMad, red) levels are slightly but significantly reduced in *AdipoR*^{27} null GSCs compared to neighboring GFP-positive controls. **(D)** Box-and-whiskers plot showing the ratio of nuclear pMad intensity in GFP-negative to that in neighboring GFP-positive GSCs in control and *AdipoR*^{27} mosaic germaria. Total numbers of GSC pairs analyzed are included below. White lines indicate averages and points represent outliers outside of the 95% confidence interval. *, $P \leq 0.05$; ***, $P \leq 0.001$, Student's *t* test. **(E-H)** The expression of Nanos (red; greyscale image shown in inset, **E**), Bruno (red; F), A2BP1 (red; G), and Orb (red; H) is unperturbed in *AdipoR*^{27} GFP-negative compared to neighboring GFP-positive GSCs in mosaic germaria. GSCs are outlined. Arrowheads indicate GFP-negative *AdipoR*^{27} null GSCs. Arrow indicates normal expression of Orb in GFP-negative *AdipoR*^{27} cyst in region 2B. GFP (green) labels wild-type cells; 1B1 (blue) labels fusomes; LamC (blue) labels cap cell nuclear envelopes, except in **(**H**)**, where 1B1 and LamC are labeled in magenta. Scale bars, 10 μm.


A
GFP
1B1
LamC
B
Average cap cell number
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6
4
2
0
260
131
25
0
1
2
Number of AdipoR27 GSCs
C
E-cadherin
1B1
LamC
E-cadherin
D
GFP-/GFP+ E-cadherin ratio
3
2
1
0
*
81
112
Control
AdipoR27
E
pMad
pMad
F
GFP-/GFP+ nuclear pMad ratio
2
1
0
***
56
84
Control
AdipoR27
G
Nanos
Nanos
H
Bruno
I
A2BP1
J
1B1
LamC
Orb

junction between cap cells and GFP-negative *AdipoR*^{27} or neighboring control GFP-positive GSCs within mosaic germaria (Figure 3.8C, D). Control mosaics showed no difference in E-cadherin intensity between GFP-negative and –positive GSCs (Figure 3.8D). In contrast, E-cadherin levels at the cap cell-GSC junction showed a subtle, but statistically significant reduction in *AdipoR*^{27} GSCs relative to neighboring wild-type control GSCs (Figure 3.8C, D). E-cadherin levels vary with the fusome cycle (Hsu and Drummond-Barbosa 2009); nonetheless, we obtained identical results by restricting our analysis to GSCs with round fusomes (Control: 0.97 $\pm$ 0.03, n = 34 pairs of GFP-negative and neighboring GFP-positive GSCs; *AdipoR*^{27}: 0.85 $\pm$ 0.03, n = 65, $P < 0.05$), ruling out any data distortion related to the fusome cycle. We therefore conclude that *AdipoR* has a minor role in GSC-cap cell adhesion.

AdipoR signaling might contribute to GSC maintenance by modulating the ability of GSCs to respond to niche-derived BMP ligands, which repress GSC differentiation (Xie and Spradling 1998). To test this possibility, we measured the levels of nuclear phosphorylated Mad (pMad), a reporter of BMP signaling (Kai and Spradling 2003) in GFP-negative relative to GFP-positive GSCs in control and *AdipoR*^{27} mosaic germaria (Figure 3.8E, F). In control mosaics, GFP-negative and neighboring GFP-positive GSCs have similar pMad levels (Figure 3.8F). *AdipoR*^{27} GSCs, however, show a small but significant reduction in pMad levels compared to neighboring GFP-positive GSCs (Figure 3.8E, F). Wild-type pMad levels do not vary during the cell cycle or with changes in fusome morphology (GSCs with round fusomes: 26.1 $\pm$ 1.36 arbitrary units [a.u.], n = 66; GSCs with non-round fusome morphologies: 23.3 $\pm$ 1.50 a.u., n = 40, P = 0.16), ruling out that the difference in pMad levels between control and *AdipoR*^{27} GSCs might be due to sampling bias. These results

therefore indicate that *AdipoR* function is required for full receptivity of GSCs to BMP ligands.

The contributions of *AdipoR* to proper E-cadherin and BMP signaling levels in GSCs are modest relative to the robust requirement for *AdipoR* in GSC maintenance, prompting us to examine whether premature expression of differentiation factors in $AdipoR^{27}$ GSCs might promote their loss. We compared the expression of Nanos, Bruno, the *Drosophila* homolog of mammalian ataxin 2-binding protein 1 (A2BP1), and Orb in GFP-negative null $AdipoR^{27}$ GSCs relative to that in neighboring GFP-positive control GSCs (Figure 3.8G-J; 10 to 25 pairs of GSCs analyzed for each marker). Nanos expression in the germarium is highest in mid-stage cysts, with low levels present in GSCs (Forbes and Lehmann 1998). Nanos expression is unperturbed in $AdipoR^{27}$ GSCs (Figure 3.8G), indicating that aberrant regulation of Nanos is not responsible for $AdipoR^{27}$ GSC loss. We also did not observe precocious expression of germline factors usually restricted to more developed germline cysts in $AdipoR^{27}$ GSCs, including the cell cycle regulator Bruno (Sugimura and Lilly 2006) and the intermediate cyst differentiation marker A2BP1 (Tastan et al. 2010) (Figure 3.8H, I). Additionally, Orb expression, usually restricted to the designated oocyte in developing germline cysts (Lantz et al. 1992), appeared normal in $AdipoR^{27}$ germline cysts (Figure 3.8J). Thus, uncovering the major contribution of *AdipoR* to GSC maintenance will require future studies thoroughly investigating a much wider range of possible molecular mechanisms.

Germline overexpression of *AdipoR* inhibits GSC loss over time

As *Drosophila* age, GSCs are lost from the niche (Xie and Spradling 1998, Pan et al. 2007). *AdipoR* is cell autonomously required for GSC maintenance (Figure 3.5), and

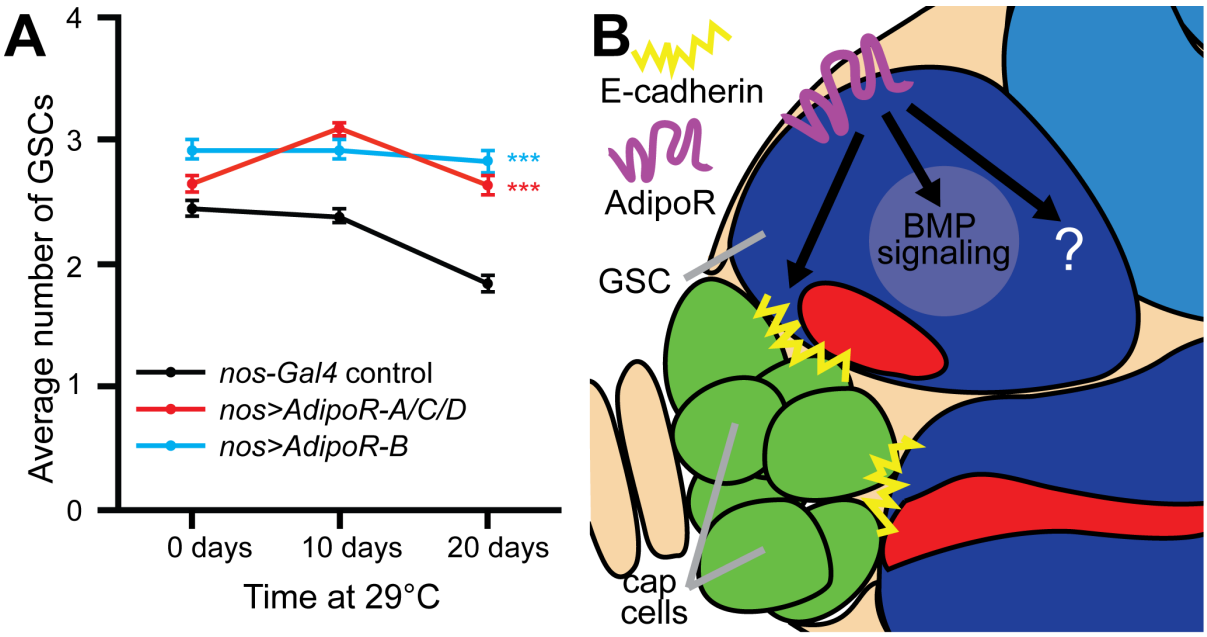


Figure 3.9. Germline overexpression of *AdipoR* counteracts normal GSC loss over time. **(A)** Females expressing either *UAS-AdipoR-A/C/D/E* or *UAS-AdipoR-B* transgenes in the germline driven by *nanos-Gal4::VP16* (*nos-Gal4*) maintain GSCs better than control females carrying the *nos-Gal4* alone. Newly eclosed females raised at 18°C were switched to 29°C for the indicated number of days. At least 70 germaria were scored for each time point. Error bars represent S.E.M. ***$P \leq$ 0.001, Two-way ANOVA with interaction. **(B)** Model for how *AdipoR* controls GSC maintenance independently of insulin signaling. *AdipoR* acts intrinsically within GSCs to promote GSC maintenance, unequivocally demonstrating that *AdipoR* controls GSCs independently of any direct effects on insulin signaling, which acts instead in cap cells to maintain GSCs (Hsu and Drummond-Barbosa 2009). Despite its major role in GSC maintenance, the contribution of AdipoR to the modulation of BMP signaling and E-cadherin levels at the GSC-cap cell junction is relatively small, suggesting that additional unknown effectors of AdipoR signaling are likely major players.

overexpression of adiponectin receptors has been shown to enhance adiponectin signaling in mammals (Luo et al. 2013, Chou et al. 2014). We therefore wondered if increasing the expression of *AdipoR* in the germline might counteract the normal process of GSC loss over time. Indeed, *nanos-Gal4::VP16*-driven germline overexpression of either *AdipoR-A/C/D/E* or *AdipoR-B* was sufficient to reverse the GSC loss observed in control Gal4 females on a rich diet (Figure 3.9). On a poor diet, GSC loss was only partially prevented by overexpression of either AdipoR isoform (Figure 3.9B), consistent with the fact that multiple diet-dependent pathways control GSC maintenance (Ables and Drummond-Barbosa 2010, Ables et al. 2012, Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, LaFever and Drummond-Barbosa 2005, LaFever et al. 2010). These results suggest that a decline in AdipoR signaling may contribute to the normal loss of GSCs that occurs over time.

Discussion

Adiponectin signaling has been reported to control progenitor cells and promote tissue regeneration (Chiarugi and Fiaschi 2010, Fiaschi et al. 2009, DiMascio et al. 2007, Shibata et al. 2008, Fiaschi et al. 2014), although it had remained unknown whether this role is linked to the insulin-sensitizing effects of adiponectin. In this study, we demonstrate in a highly tractable genetic model organism that adiponectin signaling is intrinsically required for stem cell maintenance independently of insulin-sensitization. *AdipoR* is cell autonomously required for GSC maintenance in the *Drosophila* ovary, presumably in part through enhancement of BMP signaling and E-cadherin-mediated adhesion to the niche, although additional mechanisms are likely involved (Figure 3.9B).

Surprisingly, *AdipoR* is not required to sensitize germ cells in the *Drosophila* ovary to

ILPs for the control of GSC proliferation, cyst division and growth, and vitellogenesis. Moreover, the cell autonomous role of *AdipoR* in GSC maintenance is clearly independent of insulin signaling, which is instead indirectly required in cap cells to maintain normal GSC numbers in the niche (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011). We therefore speculate that the requirement for *AdipoR* in ovarian GSC maintenance might reflect an ancient role of adiponectin receptors, with insulin sensitization representing a more recently acquired function during their evolution.

The role of *AdipoR* in GSC maintenance appears to partially depend on diet, as the increase in GSC loss relative to controls in response to *AdipoR* loss-of-function on a rich diet is more severe than on a poor diet. A potential mechanism linking *AdipoR* function to diet might be at the level of production of its yet-unidentified ligand(s) in *Drosophila*. Likewise, such ligand(s) may also be regulated with age, given that overexpression of AdipoR isoforms can revert normal GSC loss over time. The *Drosophila* genome does not encode an obvious homolog of adiponectin based on primary sequence, suggesting that the AdipoR ligand(s) has conserved three-dimensional structure in the absence of sequence conservation. In fact, osmotin, a plant ligand for the adiponectin receptor, has less than 10% sequence identity to mammalian adiponectin, but has similar tertiary structure and molecular function (Narasimhan et al. 2005). It will also be important to determine the source, in addition to the identity and regulatory mechanisms, of the AdipoR ligand(s). While adiponectin is the most abundant transcript in human adipocytes (Maeda et al. 1996), there is evidence that adiponectin is not produced strictly in adipocytes (Delaigle et al. 2006, Krause et al. 2008), raising the possibility that the *Drosophila* functional ortholog of adiponectin could be produced in other tissues.

Another focus for future investigation should be how adiponectin receptor signaling regulates stem cell maintenance. *Drosophila AdipoR* function in GSCs provides a small contribution towards full levels of E-cadherin at the niche junction and of BMP signaling. *Lissencephaly-1*, which is intrinsically required for GSC maintenance, also regulates both BMP signaling and E-cadherin levels in ovarian GSCs (Chen et al. 2010). In the *Drosophila* testis, E-cadherin trafficking and BMP signaling are linked (Michel et al. 2011). Aging female GSCs have reduced E-cadherin and BMP signaling levels (Pan et al. 2007), and we find that overexpression of AdipoR isoforms can reverse the normal GSC loss that occurs over time. It will therefore be interesting to determine whether regulation of BMP signaling and E-cadherin downstream of *AdipoR* occur separately or as part of the same signaling axis. Nevertheless, it is unlikely that the slight reductions in BMP signaling and E-cadherin levels measured in *AdipoR* null GSCs fully account for their markedly increased rate of loss. While BMP signaling controls both the self-renewal and proliferation of GSCs (Xie and Spradling 1998), AdipoR signaling is not intrinsically required for GSC proliferation (Figure 3.3), suggesting that the primary target of AdipoR is not the BMP pathway. Future studies addressing additional molecular mechanisms controlling GSC maintenance downstream of AdipoR should yield useful information that is potentially applicable to understanding the role of mammalian adiponectin signaling in precursor cells.

CHAPTER IV

AMPK HAS NUTRIENT-DEPENDENT AND –INDEPENDENT ROLES IN *DROSOPHILA* OOGENESIS

Introduction

Successful gametogenesis requires coordination of germ cell development with organismal physiology. Therefore, organisms have ancient and highly-conserved mechanisms to regulate germline activity in accordance with developmental timing, stress, and nutritional status (see Chapter I). While work in our lab and by others has demonstrated roles for many diet-dependent pathways in regulating *Drosophila* oogenesis, including those intrinsic to the ovary and through other organs (reviewed in Chapter I), we do not yet understand the full complement of ovary-intrinsic signals that mediate the ovarian response to diet. In this chapter, I describe our progress toward understanding the role of adenosine monophosphate activated kinase (AMPK), a highly conserved sensor of cellular energy, in *Drosophila* oogenesis.

AMPK is a heterotrimeric protein that controls metabolism and the cell cycle in response to cellular energy levels. AMPK activity increases with high cellular levels of adenosine mono- or diphosphate (AMP and ADP) and upon phosphorylation by its upstream kinases liver kinase B1 (LKB1) and calmodulin-dependent protein kinase kinase β (CAMKKβ) (reviewed in Hardie et al. 2016). Furthermore, high levels of ATP inhibit AMP-induced AMPK activation (Corton et al. 1995). Activated AMPK promotes catabolic processes and cell cycle arrest and inhibits anabolic processes, thus restoring energetic balance to cells with low levels of ATP (reviewed in Hardie 2015, Hardie and Ashford

2014). However, energy-independent AMPK activation occurs in response to reactivate oxygen species (Mungai et al. 2011), and CAMKKβ can promote AMPK activation without elevated AMP (Woods et al. 2005, Hurley et al. 2005, Hawley et al. 2005), indicating that specific cellular conditions might promote AMPK activation through non-canonical pathways. Many studies of AMPK have been conducted in cell culture; exploring AMPK function in a physiological context will shed light on its function in specific cell types within particular tissues.

The *Drosophila* ovary has a complex response to diet (described in Chapter I). Each ovary comprises subunits of chronologically arranged arrays of developing follicles, each consisting of germline cysts encapsulated by follicle cells, called ovarioles. Both germline cysts and follicle cells arise from stem cell populations in the germarium, the anterior portion of each ovariole (see Figure 1.1 for diagram). Germline stem cells (GSCs) divide asymmetrically to generate daughter cells, called cystoblasts, that divide four additional times to form 16-cell germline cysts. Follicle stem cells (FSCs) positioned in mid-germarium give rise to follicle cells, which divide and differentiate, encapsulating developing germline cysts as they bud off of the germarium (Margolis and Spradling 1995). Follicle cells encapsulate germline cysts in a monolayer and undergo two sequential programs of replication—the mitotic cycle, which lasts from stages 2-6, and the endocycle from stages 7-10A (Spradling 1993)—the latter of which overlaps with late stage amplification of some genes, including those encoding chorion for the egg shell (Calvi et al. 1998, Orr-Weaver 1991).

In *Drosophila*, a single gene encodes each AMPK subunit, making flies an excellent system to interrogate AMPK function. While previous studies have shown an intrinsic

requirement for AMPK in promoting follicle cell growth (Haack et al. 2013), AMPK has not been fully characterized in *Drosophila* oogenesis. Here, I characterize the role of AMPK in the ovarian response to diet using a series of independently generated mutant alleles of the *AMPK* catalytic subunit, *AMPKα*. *AMPK* intrinsically modulates follicle and GSC proliferation in response to diet and controls follicle cell growth. Furthermore, it non-autonomously controls germline cyst growth though its actions in follicle cells. We also describe a role for AMPK in promoting GSC maintenance, although its function appears to be limited to a rich diet. Finally, we also found that follicle cells intrinsically require AMPK to properly encapsulate the budding germline and that this function of AMPK is independent of diet. This study emphasizes the tissue- and cell-type specific roles for a highly conserved, nutrient-dependent signaling pathway.

Materials and Methods

Drosophila strains and culture conditions

Fly stocks were maintained on standard cornmeal/molasses/yeast/agar medium at 22-25ºC. For experiments, females (in the presence of wild-type males) were transferred daily onto either standard medium supplemented with wet yeast paste ("rich diet") or molasses/agar ("poor diet") (Drummond-Barbosa and Spradling 2001). All *AMPK* mutant stocks were maintained with a Y chromosome containing a duplication containing the *AMPKα* locus. The $AMPK^{D2}$, *FRT19A* allele was a gift from Jongkyeong Chung (Lee et al. 2007). The $AMPK\alpha^{1}$ allele was a gift from Vincent Mirouse (Haack et al. 2013), and the

AMPKα^{1} *FRT19A* recombinant chromosome was generated by standard crosses. Other genetic elements are described in FlyBase (http://www.flybase.org).

Genetic mosaic analysis

Females of genotype *y,w,His2Av-GFP hs-FLP FRT19A/AMPK* FRT19A* were generated through standard crosses. (*AMPK** represents null or wild-type alleles of the *AMPK* gene.) Zero-to 3 day-old females were maintained on dry yeast and heat shocked twice daily at 37°C for 3 days to induce mitotic recombination (Xu and Rubin 1993). For GSC maintenance assays, flies were kept on a rich diet for 3 days after the final heat shock, then either maintained on a rich diet or shifted to a poor diet for an additional 4 days prior to dissection and processing. *AMPK** homozygous clones were identified by the absence of green fluorescent protein (GFP), as detected by antibody staining, and GSCs were identified based on their anterior location and typical fusome morphology (Hsu et al. 2008, de Cuevas and Spradling 1998). To quantify GSC loss, we analyzed all germaria containing GFP-negative cystoblasts and/or cysts, and calculated the percentage of germaria that no longer contained GFP-negative GSCs (i.e. "GSC loss events"), as described (Chapter II). Budding defects were qualitatively determined by evaluating follicle cells in region 3 of mosaic germaria. Follicle growth was qualitatively assessed by comparing follicle sizes to that of flanking follicles in the same ovariole. For follicle cell size analysis, egg chambers were staged based on size, nuclear morphology, and yolk uptake (Spradling 1993). The average size of follicle cells was determined by measuring the area of a GFP-positive or GFP-negative clone with ImageJ in a single follicle cell monolayer and dividing by the number of cells in that clone. Follicles were considered misencapsulated if they contained two germline cysts contained by the same follicle cell monolayer.

To measure GSC and follicle cell proliferation, flies were maintained on a rich diet for 4 days following the last heat shock, then either switched to a poor diet or maintained on a rich diet for an additional 3 days. EdU incorporation assays were performed as described (Ables and Drummond-Barbosa 2013). The number of EdU-positive, GFP-negative GSCs/FCs was calculated as a percentage of the total number of GFP-negative GSCs/FCs as described (Chapter II).

Immunofluorescence and microscopy

Adult ovaries were dissected in Grace's Insect Medium (Lonza), teased apart, and fixed for 13 min in 5.3% fomaldehyde (Ted Pella) in Grace's. Samples were rinsed and washed four times in 0.1% Triton X-100 (Sigma) in phosphate-buffer saline (PBS), or PBT, and blocked for at least 3 h at room temperature or overnight at 4 °C in 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (NGS; Jackson ImmunoResearch) in PBT unless otherwise noted. Samples were incubated at 4 °C overnight with primary antibodies in blocking solution at the following concentrations: mouse anti-Hts (1B!) (DSHB, 1:10); mouse anti-Lamin C (LC28.26) (DSHB, 1:100); chicken anti-GFP (1:2000, Abcam); rabbit anti-pAMPK (1:200, Cell Signaling). After primary antibody incubation, samples were washed for 2 h in PBT and incubated for 2 to 4 h in Alexa Fluor 488-, 568-, or 633-conjugated goat species-specific secondary antibodies (1:200, Invitrogen). Samples were mounted in Vectashield with DAPI (Vector Laboratories). Confocal images were acquired using a Zeiss LSM 700 microscope, and analyzed using either Zeiss ZEN 2009 or ImageJ software, and equally and minimally enhanced via histogram using Adobe Photoshop CS4.

EdU incorporation assays were performed as described (Ables and Drummond-Barbosa 2013). Briefly, ovaries were dissected in Grace's medium at room temperature, and

incubated in 100 mM EdU (Invitrogen) in Grace's medium for 1 h prior to being teased apart, fixed, and stained as above. EdU was detected with AlexaFluor-594 via Click-It chemistry using manufacturer's instructions (Invitrogen) following secondary antibody incubation.

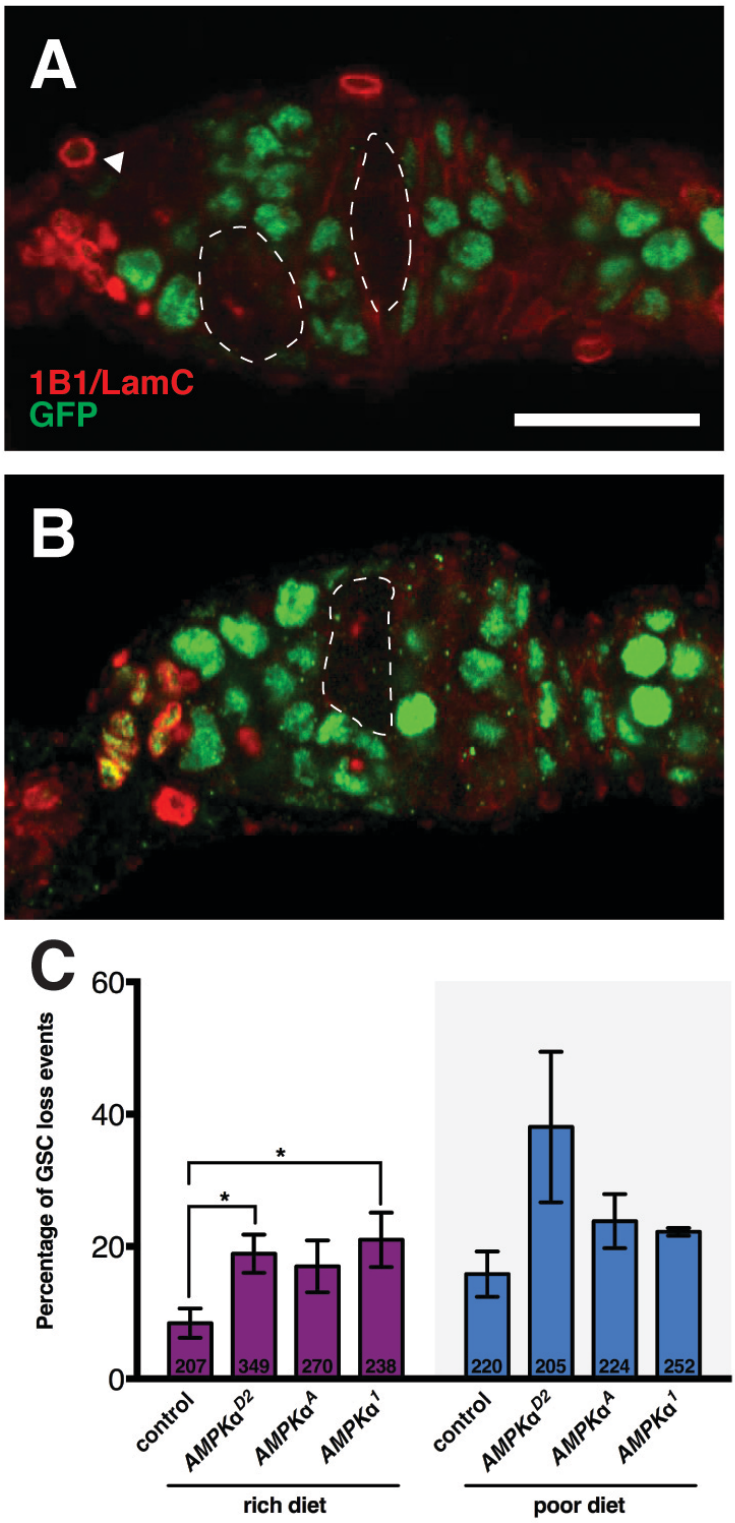
Results

Cells in the ovary intrinsically require several nutrient-sensing pathways for their function, and AMPK is a downstream effector of many of those pathways (reviewed in Chapter I, see Figure 1.2). Is AMPK a mediator of the ovarian response to diet? We acquired three independently produce alleles of the catalytic subunit of *AMPK*, *AMPK*α, and used them to generate genetic mosaic females. We then asked if any of the diet-dependent processes in the ovary were perturbed in AMPK mutant clones, which are recognized by their lack of GFP.

AMPK appears to be required for GSC maintenance in well-fed flies

When flies are fed a poor diet, GSCs are frequently lost from the niche, in part due to reduced TOR activity (LaFever et al. 2010, Sun et al. 2010) and the non-autonomous effects of systemic insulin (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, Yang et al. 2013) and ecdysone signaling (Ables and Drummond-Barbosa 2010). To determine if AMPK is intrinsically required for GSC maintenance, we compared the incidence of GSC loss events in control and AMPK mutant mosaic germaria on both rich and poor diets (Figure 4.1A-B). In control mosaics, where all cells are wild-type, GFP-negative GSCs are lost from the niche in about 10% of germaria with a mosaic germline (Figure 4.1C). *AMPK* mutant GSCs are lost approximately twice as frequently using three different mutant alleles, and *AMPK*α^{D2} and *AMPK*α^{1} mutant GSCs are lost significantly more often,

Figure 4.1. AMPK is required intrinsically for GSC maintenance on a rich diet. (A and B) Genetic mosaic germaria showing GFP-negative germline cystoblasts and cysts (outlined) are clonally derived from GFP-negative GSCs (arrowhead) (A). The presence of GFP-negative germline cysts without a GFP-negative GSC indicates a GSC loss event (B) 7 days after clone induction. GFP (green) labels wild-type cell nuclei; 1B1 (red) labels fusomes and cell membranes; LaminC (LamC; red) labels cap cell nuclear envelopes. Scale bar, 10 μm. **(C)** Quantification of GSC loss events in control and *AMPK* mutant mosaic germaria on rich and poor diets show significant loss of *AMPK* mutant GSCs at 7 days after clone induction on a rich, but not on a poor, diet. Sample sizes from 4 independent trials are indicated in each bar. Error bars represent S.E.M. **P*<0.05 by Student's *t* test.


A
1B1/LamC
GFP
B
C
Percentage of GSC loss events
60
40
20
0
*
*
207
349
270
238
220
205
224
252
control
AMPKα D2
AMPKα A
AMPKα 1
control
AMPKα D2
AMPKα A
AMPKα 1
rich diet
poor diet

on a rich diet. When flies are shifted to a poor diet, however, *AMPK* mutant GSCs are lost at a comparable rate to controls. Since *AMPK* mutant GSCs are lost from the niche on a poor diet, their mechanism for sensing the nutritional environment is intact, demonstrating that *AMPK* is not required for poor diet-induced GSC loss. Based on its role as a nutrient sensor in the literature (Gowans and Hardie 2014, Hardie 2014, Hardie and Ashford 2014, Hardie and Hawley 2001, Hardie et al. 2012, Hardie et al. 2016), AMPK activity is expected to be lowest when flies are fed a rich diet and increase in response to poor diet. In contrast, these data suggest that basal level *AMPK* activity is required in GSCs to promote maintenance under normal dietary conditions.

AMPK may control GSC proliferation in response to diet

When flies are fed a poor diet, oogenesis decreases its pace uniformly, and GSC proliferation slows down (Drummond-Barbosa and Spradling 2001). Previous work from our lab demonstrated a direct requirement for insulin-like peptides (ILPs), TOR, and ecdysone in GSC proliferation (LaFever and Drummond-Barbosa 2005, LaFever et al. 2010, Ables and Drummond-Barbosa 2010). These known regulators have high activity when flies are well-fed and stimulate proliferation; it would be interesting to know, however, whether active repression of proliferation is important on a poor diet. We reasoned that AMPK, a nutrient sensor downstream of insulin signaling (see Figure 1.2), might be required to mediate this diet-induced suppression of the cell cycle. To test this, we incubated *AMPK* mutant mosaic ovarioles with the thymidine analog EdU, a marker for S phase (Figure 4.2A-B). Indeed, the frequency of control mosaic GSCs in S phase decreases by approximately fivefold when flies are shifted from a rich to a poor diet (Figure 4.2C). This change, however, is not observed in *AMPK* mutant mosaic flies, and *AMPK* mutant GSCs incorporate

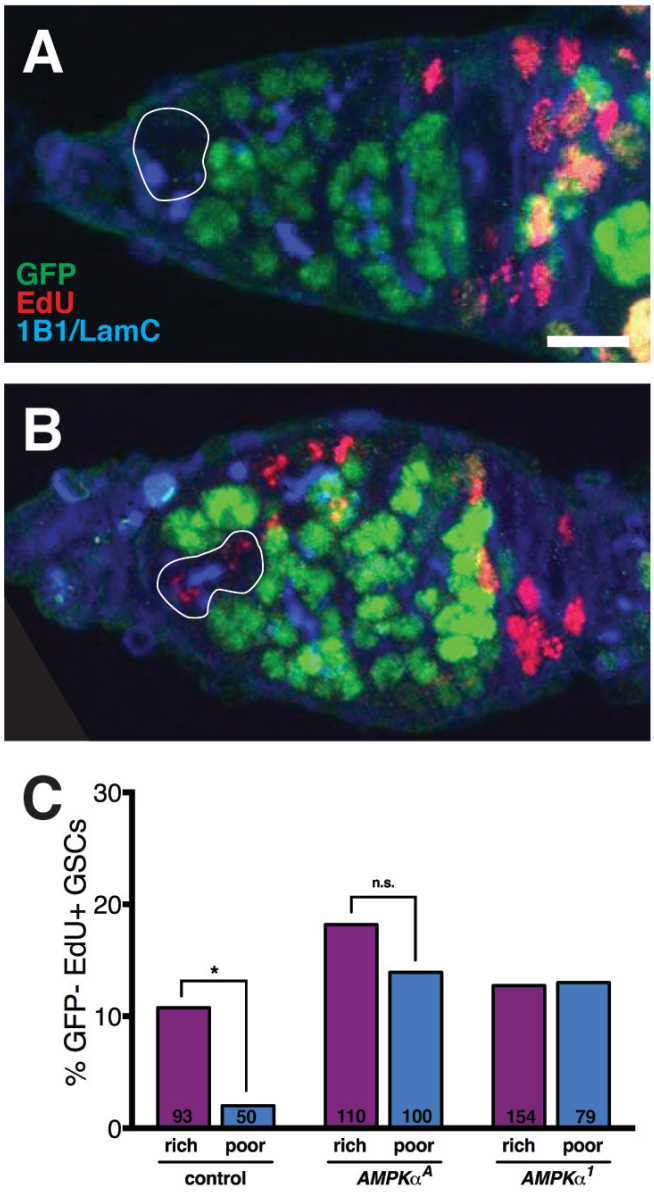


Figure 4.2. AMPK may be required to control GSC proliferation in response to diet. (A and B) Maximum intensity projections of genetic mosaic germaria showing GFP-negative GSCs (outlined) without (A) and with (B) EdU incorporation 7 days after clone induction. GFP (green) labels wild-type cell nuclei; 1B1 (blue) labels fusomes and cell membranes; LaminC (LamC; blue) labels cap cell nuclear envelopes; EdU (red) labels nuclei in S phase. Scale bar, 10 μm. **(C)** Average percentage of GFP-negative GSCs in control and *AMPK* mutant mosaic germaria that incorporate EdU. These data represent two independent trials, and sample size is indicated in each bar. **P*<0.05 by Chi-square analysis.

EdU in similar proportions regardless of diet. Indeed, the frequency of EdU incorporation in *AMPK* mutant GSCs is statistically indistinguishable from that of control mosaics regardless of diet (Figure 4.2C), consistent with a requirement for *AMPK* in repressing cell cycle progression in response to poor diet. Further analysis with additional cell cycle markers will determine what cell cycle phase(s) are regulated by AMPK.

AMPK is required cell-autonomously by follicle cells, but non cell-autonomously-for germline cyst growth

AMPK appears to act as a nutrient sensor in ovarian GSCs, regulating their proliferation in response to diet. Because AMPK restricts cellular growth in times of nutrient deprivation (Yuan et al. 2013), we evaluated *AMPK* mutant cell growth in flies fed both rich and poor diets. TOR signaling, which is negatively regulated by AMPK in many contexts (Hindupur et al. 2015), is intrinsically required by both the germline and its surrounding follicle cells for germline cyst growth (LaFever et al. 2010). Surprisingly, AMPK is dispensable in the germline for follicle growth. In both control (Figure 4.3A) and *AMPK* mutant mosaic ovarioles (Figure 4.3B) follicles with mutant, GFP-negative germline cysts develop at normal rates compared to flanking GFP-positive follicles. Intriguingly, AMPK is required intrinsically in follicle cells to control the growth of the underlying germline cyst (Figure 4.3C). While this is never observed in control mosaics, ovarioles with *AMPK* mutant follicle cells sometimes contain cysts that develop at a faster rate than the older, posterior germline follicles (Figure 4.3D). Precociously growing cysts often contain large patches of *AMPK* mutant follicle cells. When flies are fed a rich diet, cyst overgrowth occurs in approximately 10% of ovarioles with mosaic follicle cells; culturing the flies on a poor diet,

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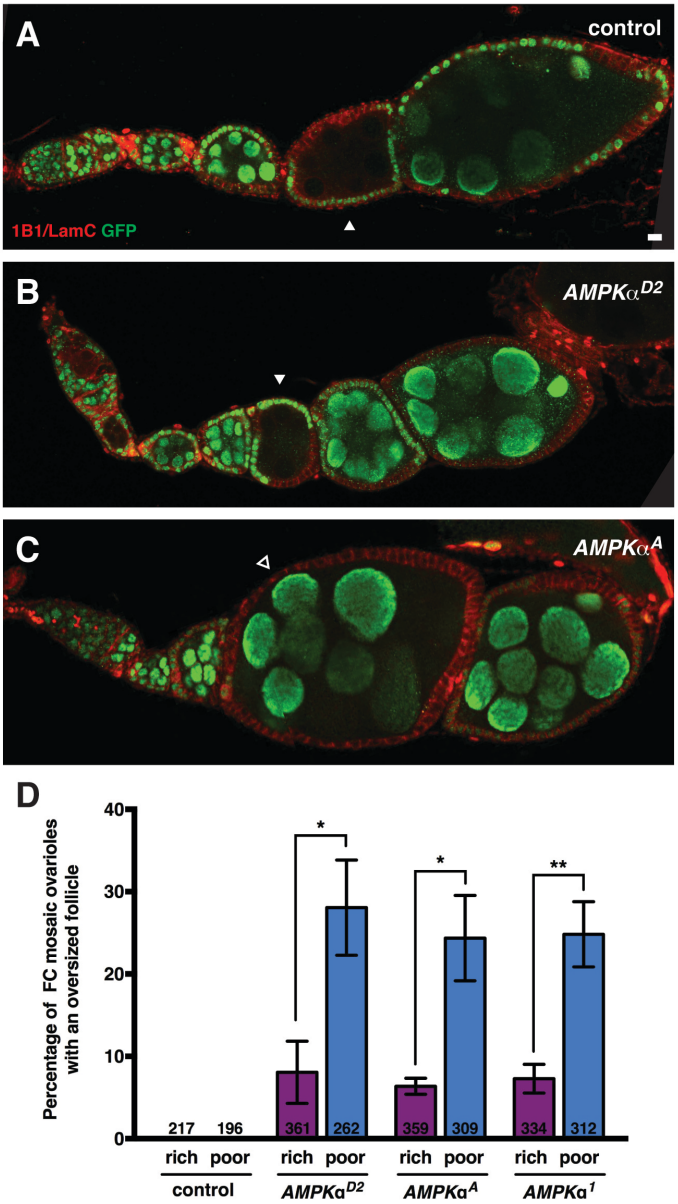


Figure 4.3. AMPK non-autonomously controls germline cyst growth via follicle cells, but not intrinsically. (A and B) Control (A) and *AMPK* mutant mosaic (B) ovarioles with GFP-negative germline cysts (arrowheads) that grow normally relative to flanking GFP-positive cysts. **(C)** *AMPK* mutant mosaic ovariole with a GFP-negative follicle cell layer has an underlying germline cyst (open arrowhead) that is larger than the posterior, older germline cyst, which is also GFP-positive and is surrounded by fewer GFP-negative follicle cells. Anterior is to the left. GFP (green) labels wild-type cell nuclei; 1B1 (red) labels fusomes and cell membranes; LaminC (LamC; red) labels cap cell nuclear envelopes. Scale bar, 10 μm. **(D)** Quantification of cyst growth defect in follicle cell mosaic ovarioles shows a diet-dependent effect of *AMPK* in non-autonomously controlling germline cyst growth 7 days after clone induction. Sample sizes are shown in each bar and indicate results from three independent trials. **P*<0.05; ***P*<0.01 by Student's *t* test.

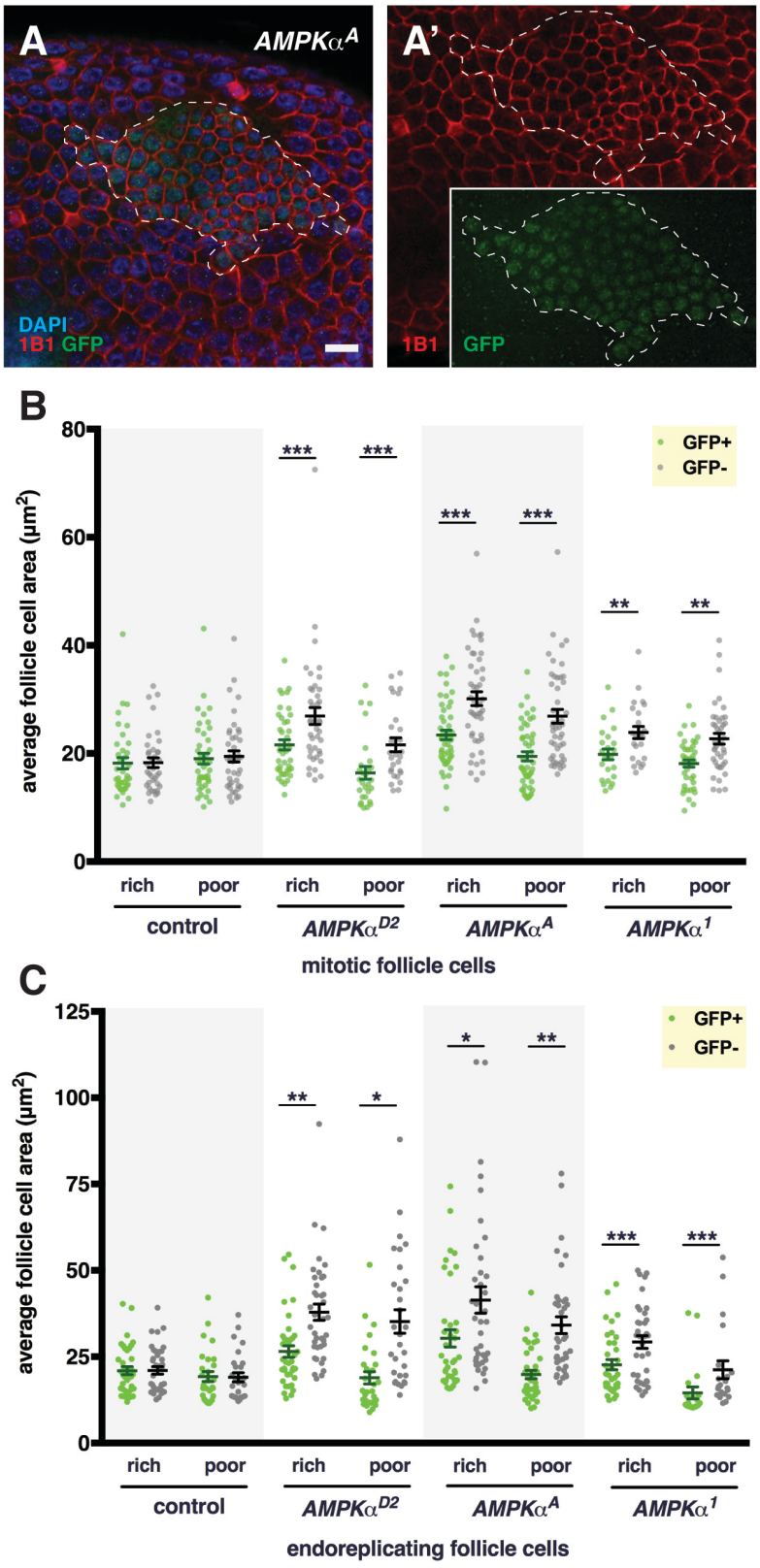
however, triples the penetrance of this phenotype. Therefore, while AMPK does not cell-autonomously control germline cyst growth, it does so non-autonomously via follicle cells.

AMPK controls follicle cell size in mitotic and endoreplicating follicle stages and may control mitotic follicle cell proliferation

Overactivation of follicle cell growth or proliferation could each be responsible for the misregulated germline cyst growth in *AMPK* mutant mosaic ovarioles. Indeed, in late stage follicles, where follicle cells undergo endoreplication (Spradling 1993), we often observe wild-type, GFP-positive clones with markedly smaller follicle cells than neighboring, GFP-negative follicle cells (Figure 4.4A-A'). We went on to compare the cell areas of neighboring GFP-negative and GFP-positive follicle cell clones in control and *AMPK* mutant mosaic ovarioles on both rich and poor diets. While in control mosaic ovarioles, GFP-negative and GFP-positive follicle cells are comparably sized, *AMPK* mutant GFP-negative follicle cells are consistently larger than neighboring wild-type GFP-positive follicle cells, regardless of whether follicles were at a mitotic or endoreplicative stage of oogenesis or their dietary condition (Figure 4.4B-C). This effect is consistent with a previously published descriptions of an additional *AMPK* mutant allele, $AMPK\alpha^{3}$ (Haack et al. 2013) and of protein phosphatase V, a negative regulator of AMPK, in the larval fat body (Yin et al. 2014). We conclude that *AMPK* controls follicle cell growth under both normal and poor nutrient conditions. Since follicle cell size dramatically increases as cells enter endoreplication, it will be interesting to determine whether AMPK, like TOR (LaFever et al. 2010) regulates this switch.

Since AMPK regulates follicle cell growth under rich and poor diet conditions, this function of AMPK is unlikely to explain the threefold increase in oversized germline cysts

Figure 4.4. AMPK intrinsically controls follicle cell growth during mitosis and endoreplication. (A and A') In an *AMPK* mutant mosaic follicle cell layer, wild type, GFP-positive follicle cells (outlined; single channel in inset) are surrounded by larger mutant follicle cells. 1B1 (red) marks cell membranes; DAPI (blue) marks nuclei. Scale bar, 10 μm. **(B and C)** At 7 days after clone induction, *AMPK* mutant, GFP-negative follicle cells are larger than neighboring GFP-positive follicle cells in mitotic (B) and endoreplicating (C) follicles. Error bars represent S.E.M. **P*<0.05, ***P*<0.01; ****P*<0.001 by Student's *t* test.


A
AMPKα^A
DAPI
1B1 GFP
A'
1B1
GFP
B
average follicle cell area (µm²)
80
60
40
20
0
GFP+
GFP-
***
***
***
***
**
**
rich
poor
rich
poor
rich
poor
rich
poor
control
AMPKα^D2
AMPKα^A
AMPKα^1
mitotic follicle cells
C
average follicle cell area (µm²)
125
100
75
50
25
0
GFP+
GFP-
**
*
*
**
***
***
rich
poor
rich
poor
rich
poor
rich
poor
control
AMPKα^D2
AMPKα^A
AMPKα^1
endoreplicating follicle cells

when *AMPK* mutant ovarioles are shifted to a poor diet (Figure 4.3D). Our preliminary data implicate AMPK in the response of GSC proliferation to diet (Figure 4.2). Does AMPK likewise control follicle cell proliferation in response to diet? EdU incorporation on a rich diet is comparable between control and *AMPK* mutant follicle cells, suggesting that AMPK does not control the cell cycle *per se* (Figure 4.5). Additionally, the trends in our preliminary data suggest that, unlike control mosaic follicle cells, *AMPK* mutant follicle cells are unable to suppress proliferation in response to a poor diet (Figure 4.5). These effects mirror those we observed in GSC EdU incorporation (Figure 4.2). Sample sizes for these experiments are low, and both further trials and additional mitotic markers are necessary to definitively determine whether there is a role for *AMPK* in controlling follicle cell proliferation. Taken together, our data suggest that multiple AMPK-dependent mechanisms contribute to the non-autonomous regulation of germline growth by follicle cells.

AMPK controls follicle cell development independent of diet

The phenotypes described thus far in this chapter are diet-dependent or related to the canonical nutrient-sensing role of AMPK. Additionally, AMPK regulates follicle cell encapsulation of cysts in the germarium, a process not perturbed in the described ovarian response to diet (see Chapter I). During oogenesis, germline cysts bud off of the germarium, and a single layer of follicle cells encapsulates each 16-cell germline cyst (Figure 4.6A). *AMPK* mutant follicle cells, however, frequently fail to properly execute this budding event, and mutant follicle cells surround sacs of multiple germline cysts (Figure 4.6B). Follicle budding defects appear to occur more frequently on a poor diet (Figure 4.6D). However, the outcome of these budding defects, where multiple germline cysts are encapsulated in the same follicle cell monolayer (Figure 4.6C) is not observed more frequently on a poor diet

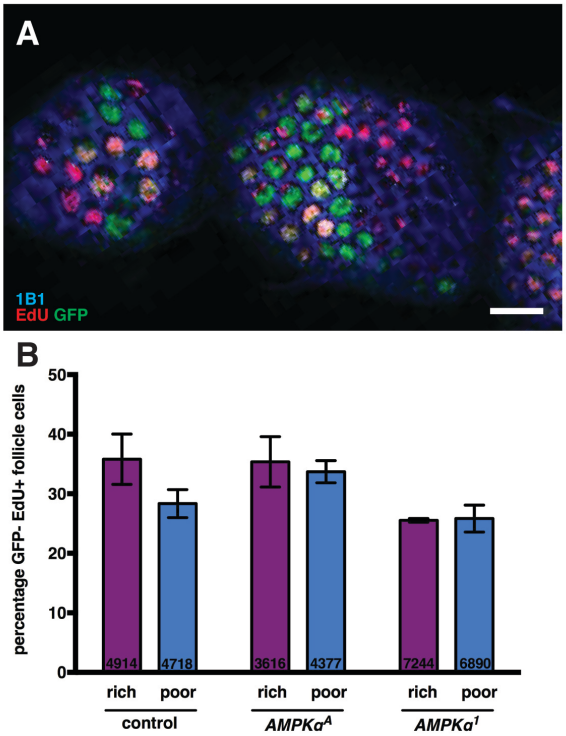
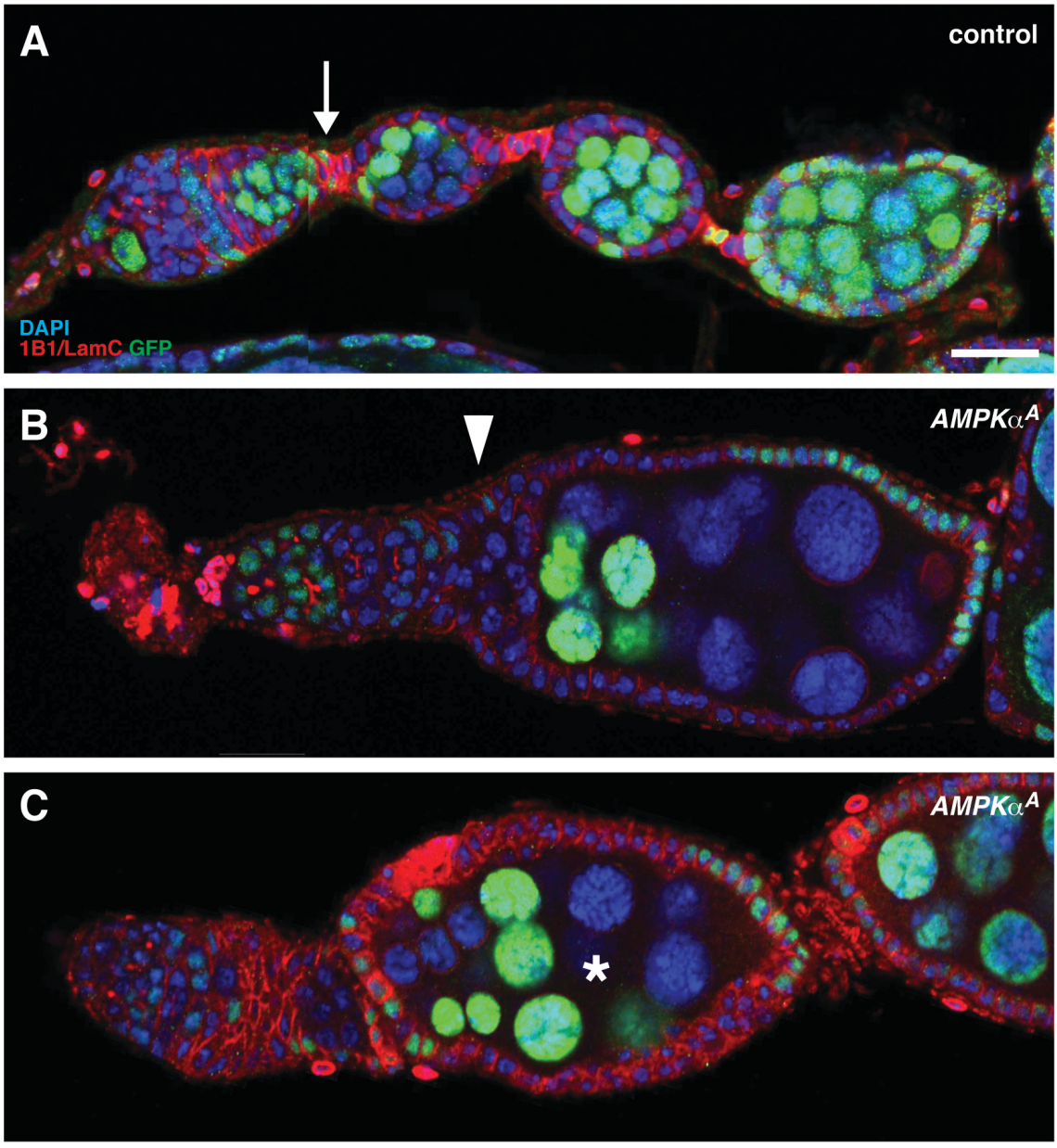
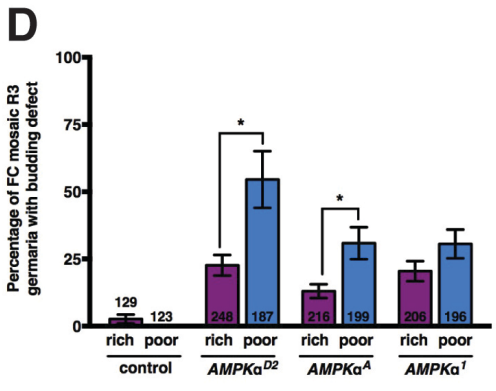
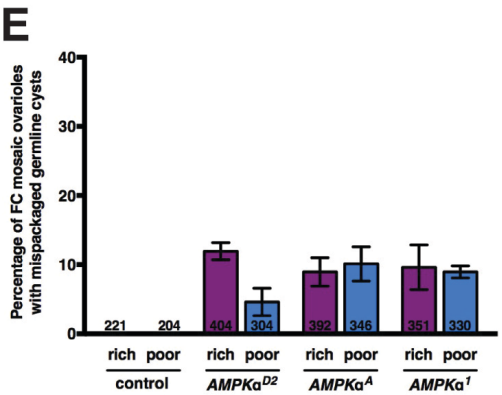


Figure 4.5. AMPK might contribute toward reduced follicle cell rates on a poor diet. **(A)** A genetic mosaic ovariole showing GFP-negative follicle cells with EdU incorporation. GFP (green) labels wild-type cell nuclei; 1B1 (blue) labels cell membranes; EdU (red) labels cells in S phase. Scale bar, 10 μm. **(B)** Average percentage of control and *AMPK* mutant mosaic GFP-negative follicle cells in mitotic stages that incorporate EdU 7 days after clone induction. Sample sizes from two independent trials are indicated in each bar. Data are not statistically significant by two-way ANOVA with interaction test.

Figure 4.6. Follicle cell AMPK controls follicle encapsulation independent of diet. (A) In control mosaic ovarioles, germline cysts bud off of the posterior germarium and form follicle cell stalks (arrow), which separate follicles for the remainder of oogenesis. **(B)** *AMPK* mutant follicle cells (arrowhead) do not support normal follicle budding in region 3 of the germarium. **(C)** *AMPK* mutant mosaic follicle cells surround a mispackaged germline cysts (asterisk) that contains nurse cells of widely variable ploidy, presumably an outcome of the event depicted in (B). GFP (green) labels wild type nuclei; 1B1 (red) labels fusomes and cell membranes; DAPI (blue) labels nuclei. Scale bar, 20 μm. **(D and E)** Graphs indicating the frequency of phenotypes shown in (B) and (C) demonstrate that, while budding defects are observed more frequently on a poor diet (D), they resolve into mispackaged follicles at the same rate on each diet (E) at 7 days after clone induction. Sample sizes represent data from four independent trials and are shown in each bar. Error bars, S.E.M. $*P<0.05$, Student's *t* test.


A
control
DAPI
1B1/LamC GFP
B
AMPKαA
C
AMPKαA
*


D
Percentage of FC mosaic R3 germaria with budding defect
100
75
50
25
0
129
123
248
187
216
199
206
196
*
*
rich poor
control
AMPKαD2
AMPKαA
AMPKα1


E
Percentage of FC mosaic ovarioles with mispackaged germline cysts
40
30
20
10
0
221
204
404
304
392
346
351
330
rich poor
control
AMPKαD2
AMPKαA
AMPKα1

(Figure 4.6E), indicating that the final process of encapsulation is resolved equally well regardless of by diet. Therefore, we hypothesize that follicle budding defects are observed more frequently on a poor diet because of the global slowing of oogenesis on a poor diet rather than a specific nutritional input. As oogenesis equally slows, the time it takes to execute follicle budding slows, and errors in that process are more evident in fixed tissue.What AMPK targets are responsible for follicle cell developmental defects? Strong candidates are Notch and hedgehog signaling, both of which have well-characterized roles in controlling follicle cell development (Forbes et al. 1996, Ruohola et al. 1991, Chang et al. 2013, Nystul and Spradling 2010). I am currently performing genetic interaction experiments to try to understand which, if either, of these signaling cascades interacts with AMPK to regulate follicle cell development.

Is AMPK required for cell survival in the *Drosophila* ovary?

A striking phenotype in AMPK mutant mosaics is the filling of the muscle sheath surrounding the ovariole with cellular debris, causing it to expand away from its normal closely juxtaposed position (Figure 4.7). This phenotype, which we call a "swollen sheath", indicates massive cell death within the ovariole. Swollen sheaths are almost never observed in control ovarioles, but are seen in approximately 30% of $AMPKa^{D2}$ mosaic ovarioles (Figure 4.7D) and are also observed in other alleles of AMPK (data not shown). Which cells in *AMPK* mutant mosaic ovarioles are dying? To assess cell death inside and outside of the germarium, I will stain genetic mosaic ovaries with an antibody against the apoptosis marker Dcp-1. This will allow us to determine whether germline, somatic tissue, or both is responsible for the swollen sheath phenotype. Future analysis will also address whether diet influences the penetrance of the swollen sheath phenotype.

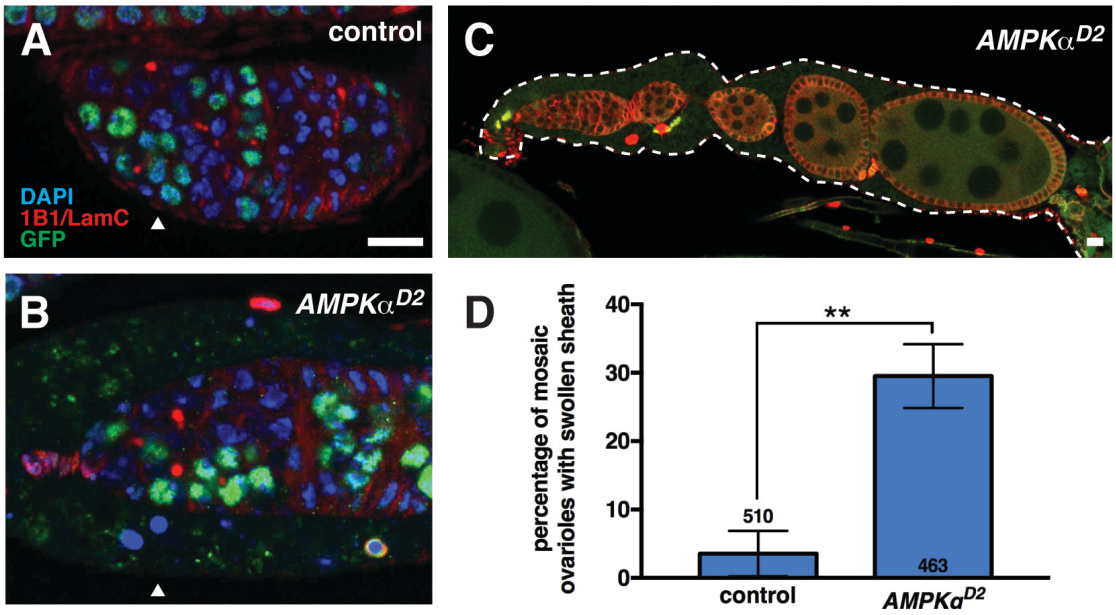


Figure 4.7. AMPK mutant mosaic ovarioles have swollen sheaths. (A) Control mosaic germaria are surrounded by a closely juxtaposed muscle sheath (arrowhead) 7 days after clone induction. **(B** and **C)** In *AMPK* mutant mosaic ovarioles, the muscle sheath (arrowhead; dashed line) is filled with cellular debris, presumably due to massive cell death at 7 (B) and 15 (C) days after clone induction. DAPI (blue) marks nuclei; 1B1 (red) labels cell membranes and fusome; LaminC (LamC; red) labels cap cells; GFP (green) labels wild-type cells. Scale bar, 10 μM; images in panel (A) and (B) were acquired at the same magnification. **(D)** Quantification of the percentage of ovarioles containing a swollen sheath in well-fed control and $AMPK^{D2}$ mosaic ovarioles at 15 days after clone induction. Sample sizes are indicated in each bar and represent the number of ovarioles quantified over three independent trials. Error bars represent S.E.M. $^{**}P<0.01$ by Student's *t* test.

pAMPK antibody is not a specific marker of activated AMPK in whole mount ovarioles

Previous studies in *Drosophila* and in cell culture have used a commercially available antibody against phosphorylated AMPKα (pAMPK) as a readout for AMPK activity in whole mount samples (Castanieto et al. 2014, Vazquez-Martin et al. 2011, Lee et al. 2015). Observations by a former student in the lab, Shaina Palmere, indicated that pAMPK staining localized to centrosomes in mitotic cells and was not reduced upon AMPK subunit knockdown (S.P., unpublished observations). Indeed, pAMPK has been widely reported to localize to mitotic poles in whole mount tissues. Because we did not detect pAMPK staining in cells outside of mitosis (data not shown), we were concerned that pAMPK antibody was not specific. To test this, we generated *AMPK* mutant mosaic germaria and looked for pAMPK-positive cells in GFP-negative, *AMPK* mutant cells. Indeed, we observed pAMPK-positive *AMPK* mutant cells (Figure 4.8); these cells were always in mitosis. As we observe this in all three mutant *AMPK* alleles (data not shown), we conclude that this pAMPK antibody is not a suitable tool to measure AMPK activity in whole mount samples.

Discussion

Work described in this chapter suggests that AMPK has distinct, but overlapping, roles in the two stem cell lineages in the *Drosophila* ovary. AMPK acts as a nutrient sensor in follicle cells to control their growth and, non-autonomously, the growth of underlying germline cyst, but is dispensable in the germline for cyst growth. *AMPK* is required for GSC maintenance on a rich, but not a poor, diet, indicating that this role for AMPK is diet-dependent; this is suggests that basal levels of AMPK are required for GSC maintenance. In its capacity as a nutrient sensor, however, AMPK slows proliferation of both GSCs and follicle cells on a poor diet. Furthermore, AMPK acts as a survival factor in the ovary, and

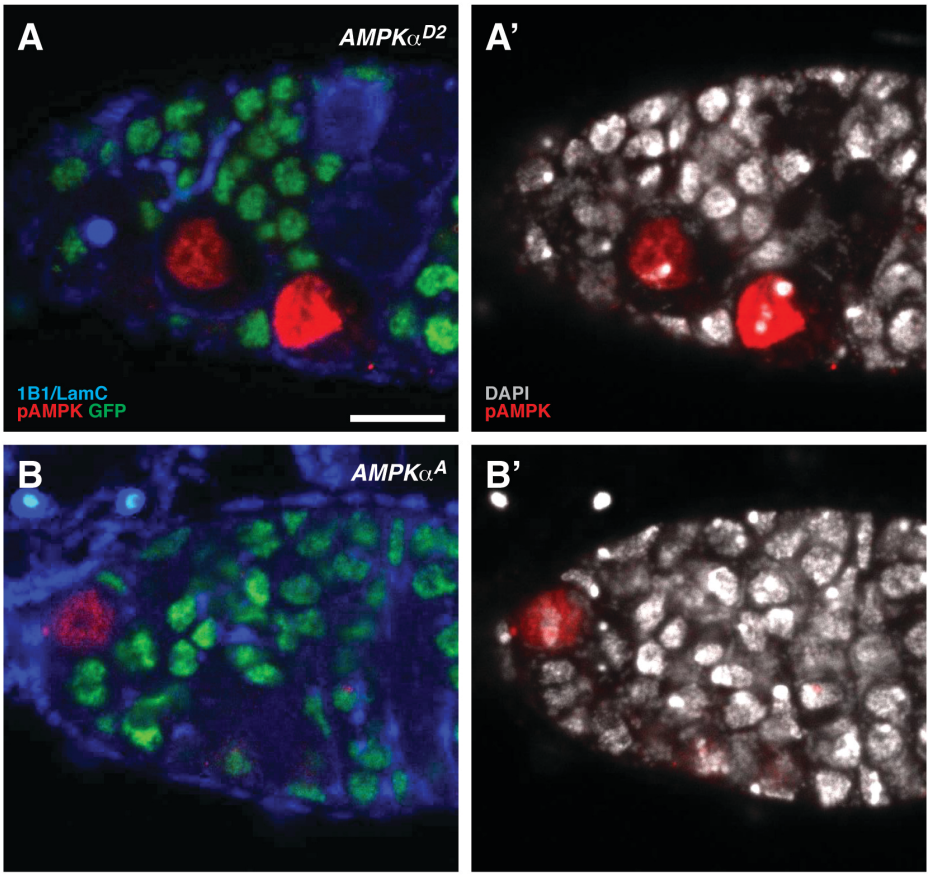


Figure 4.8. Phosphorylated-AMPK antibody does not specifically label activated AMPK in whole mount ovarian samples. *AMPKα*^{1} (A and A') and *AMPKα*^{A} (B and B') mutant germline cells in mitosis are positive for pAMPK, indicating that the antibody is not a reliable marker for activated AMPK. pAMPK (red); GFP (green), labels wild type nuclei; 1B1 (blue) labels fusome and cell membranes; LaminC (LamC; blue) labels cap cell nuclear envelopes; DAPI (grey) labels nuclei. Scale bar, 10 μm.

genetic mosaic ovarioles experience massive cell death, although whether this reflects a contribution of germline or soma to cell death remains to be seen. Finally, we described a role for AMPK in follicle cell encapsulation of germline cysts that is independent of its role as a nutrient sensor. Three independently generated alleles of *AMPK* present similar phenotypes, strongly supporting the conclusion that these phenotypes reflect *bona fide* requirements for *AMPK* in the adult *Drosophila* ovary.

AMPK may control germ and somatic cell proliferation in response to diet

Our preliminary data suggest that AMPK is required to downregulate GSC and follicle cell proliferation in response to poor diet (Figure 4.2). Similarly, the *Caenorhabditis elegans* homologs of *AMPKa*, *aak-1* and *aak-2*, suppress germline proliferation during nutrient-dependent developmental arrests, and double mutants have hyperplastic germlines (Fukuyama et al. 2012, Narbonne and Roy 2006). Failure to maintain germline quiescence during developmental nutritional arrests is catastrophic, leading to precocious entry into meiosis (Narbonne and Roy 2006) and sterility in surviving animals (Fukuyama et al. 2012). Both worm and fly roles are consistent with a role for AMPK in regulating the cell cycle in response to nutrient availability, and positions the ovary as a system in which to study the mechanism of this control.

AMPK differentially regulates ovarian germline and somatic lineages

While AMPK appears to control proliferation in response to diet in both the ovarian germline and soma, it is not required in both lineages for cell growth. Follicle cell AMPK regulates both cell-autonomous growth and the growth of the underlying germline cyst; conversely, germline AMPK is dispensable for cell growth (Figure 4.4). In the *Drosophila* ovary, somatic follicle cell growth directly regulates both the growth and developmental

stage of the underlying germline such that precociously growing germline cysts maintain developmental timing concordant with their size (Vachias et al. 2014). Germline-soma growth coordination has been investigated in the ovary in the context of normal development (Gilboa and Lehmann 2006, Lopez-Schier, 2001 #516); how diet may interact with this process, however, remains elusive (Vachias et al. 2014). Several genes control germline growth both intrinsically and via follicle cells, including *Tor* and the oncogene *dMyc* (LaFever et al. 2010, Maines et al. 2004); this is the first example, to our knowledge, of a gene that controls germline growth and development non-autonomously via follicle cells without also being intrinsically required for germline cyst growth. Understanding the distinct effectors of AMPK activity in the germline versus the somatic ovary will shed light on their differential regulation and responses to diet.

Tor is a potential mediator of diet-dependent role of AMPK

What are the targets of AMPK activity in the *Drosophila* ovary? Given the different, but overlapping, effects of removing AMPK from follicle and germline cells, there are likely different downstream effectors acting in each cell type. Target of rapamycin, or TOR, is a cellular integrator of nutritional information that acts downstream of AMPK in many systems (Hardie et al. 2016). Indeed, the roles of AMPK and TOR signaling are fundamentally linked; tuberous sclerosis complex 2 (TSC2), an upstream inhibitor of Tor, contains an AMPK phosphorylation site conserved from *Drosophila* to mammals (Kim and Lee 2015), and the kinases are functionally poised for direct or indirect cross-talk (reviewed in Hindupur et al. 2015). Does hyperactive TOR signaling due to *AMPK* deletion account for the phenotypes observed in *AMPK* mutant mosaic ovaries? In genetic mosaic ovarioles, *Tor* mutant follicle cells are smaller than the neighboring wild-type, consistent with a model in

which AMPK acts as a major upstream inhibitor of TOR signaling (LaFever et al. 2010). . Consistent with its role as a negative regulator of TOR, AMPK mutant GSCs only have perturbed proliferation under poor diet conditions, while *Tor* mutant GSCs have slowed cell cycles when well-fed (LaFever et al. 2010). In the germline, however, Tor likely receives further AMPK-independent inputs; this model is supported by the dispensability of AMPK in the germline for cyst growth. Furthermore, *Tor* and *Tsc1* mutant GSC loss is dramatic compared to what we observe in AMPK mutant mosaics(LaFever et al. 2010, Sun et al. 2010), consistent with major upstream inputs for TOR signaling outside of AMPK, including amino acid sensing. I am currently testing whether removal of a single copy of *Tsc1* or *Tor* can rescue the *AMPK* mutant ovarian phenotypes.

Notch or hedgehog signaling may mediate the developmental functions of AMPK

AMPK regulates follicle cell development independent of diet, and delays in germline cyst budding with AMPK mutant follicle cells lead to the encapsulation of multiple germline cysts (Figure 4.6). Why do AMPK mutant follicle cells fail to appropriately encapsulate germline cysts? Hedgehog (Hh) and Notch signaling, each with well-characterized roles in germline follicle budding (Forbes et al. 1996, Ruohola et al. 1991) and follicle cell specification (Chang et al. 2013, Nystul and Spradling 2010), are enticing candidates to act with AMPK in controlling follicle cell encapsulation and budding in the late germarium.

In hepatocellular carcinoma cells, AMPK negatively regulates glibolastoma-associated oncogene 1 (GLI1), a transcription factor that is a downstream activator of Hh signal transduction (Xu et al. 2014), and AMPK directly phosphorylates GLI1 in medulloblastoma cells, destabilizing it (Li et al. 2015). Therefore, AMPK acts as a negative regulator of Hh signal transduction in multiple cell types *in vitro.* If this mechanism is

conserved in *Drosophila* follicle cells, we predict that the protein encoded by the *Drosophila* homolog of *Gli1*, *Cubitus interruptus*, will be stabilized in *AMPK* mutant follicle cells, and removal of *hh* will rescue the mutant follicle cell budding defect. I am currently in the process of testing this model.

Several lines of evidence suggest that Notch and AMPK signaling could interact in the *Drosophila* ovary. A recent screen for Notch interactors in follicle cells uncovered its interaction with multiple processes associated with metabolic state, including translational machinery and genes associated with protein degradation (Jia et al. 2015). Notch signaling is also regulated by autophagy, a process downstream of AMPK (Hardie et al. 2016). While it is clear that autophagy is upregulated in ovaries from nutrient-deprived flies, autophagy is also required for oogenesis under well-fed conditions (Barth et al. 2011). Follicle cell mutant clones of *ATG1*, a major autophagy-related gene, have fused egg chambers without stalk cells, reminiscent of both *Notch* and *AMPK* mutant phenotypes (Barth et al. 2012). Furthermore, Notch controls the switch from mitosis to the endocyte at mid-oogenesis in *Drosophila* (Lopez-Schier and St Johnston 2001, Deng et al. 2001, Lilly and Duronio 2005), and the large size of *AMPK* mutant follicle cells (Figure 4.4) could reflect precocious entry into the endocycle. Taken together, these lines of evidence form a compelling case to evaluate Notch signaling in *AMPK* mutant follicle cells, and I am currently evaluating Notch target gene expression, including *cut* and *hindsight*, in *AMPK* mutant follicle cells.

CHAPTER IV

ADIPOCYTE AMINO ACID SENSING CONTROLS ADULT GERMLINE STEM CELL NUMBER VIA THE AMINO ACID RESPONSE PATHWAY AND INDEPENDENTLY OF TARGET OF RAPAMYCIN SIGNALING IN *DROSOPHILA* FEMALES

This chapter appeared in *Development* [Armstrong, Laws, and Drummond-Barbosa, *Development.* 2014 December; 141(23): 4479-4488] and is reproduced here verbatim. I performed and interpreted experiments.

Introduction

Stem cell lineages are inextricably linked to whole-body physiology and nutrient availability in multiple organisms (Ables et al. 2012). For example, diet influences wound healing, hematopoietic transplants, and cancer risk in humans, and evidence ranging from human epidemiological to model organism experimental data suggests that diet-dependent pathways impact a variety of adult stem cells (Ables et al. 2012). As intact living organisms vary their dietary input, multiple tissues and organs sense and respond to diet; however, our knowledge of how inter-organ communication contributes to the dietary control of adult stem cells remains limited.

The obesity epidemic has brought to light the critical importance of normal adipocyte function in maintaining a healthy physiology. Adipocytes are highly sensitive to diet and produce long-range factors with key roles in metabolism, reproduction, and other physiological processes (Rosen and Spiegelman 2014). Conversely, dysfunctional adipocytes underlie the link between obesity and several diseases, including cancers (Vucenik and Stains 2012). Whether sensing of dietary inputs by adipocytes leads to specific effects on adult stem cells in other organs, however, remains largely unexplored.

Drosophila female germline stem cells (GSCs) sense and respond to diet through complex endocrine mechanisms (Ables et al. 2012). Two to three GSCs reside within a well-defined niche in the germarium, the anterior portion of the ovariole (Figure 5.1A-C). Each asymmetric GSC division yields another GSC and a cystoblast that forms a 16-cell cyst, which is enveloped by follicle cells to generate a follicle that develops through oogenesis to form a mature oocyte (Spradling 1993). On a yeast-rich diet, GSCs and their progeny grow and proliferate faster than on a yeast-free diet (Drummond-Barbosa and Spradling 2001), and this response is mediated by diet-dependent factors acting on or within the ovary. For example, optimal levels of TOR activity likely controlled by circulating amino acids are intrinsically required in GSCs for their proliferation and maintenance (LaFever et al. 2010, Sun et al. 2010). Insulin-like peptides produced by medial neurosecretory cells in the brain act directly on GSCs to modulate how fast they proliferate to generate new cystoblasts (Hsu et al. 2008, LaFever and Drummond-Barbosa 2005). In parallel, insulin-like peptides act directly on cap cells, the major cellular components of the niche, to control GSC maintenance via two mechanisms. Insulin-like peptides promote the response of cap cells to Notch ligands (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011), which are required for proper cap cell numbers (Song et al. 2007)}, and also GSC-cap cell attachment via E-cadherin (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011). These past studies, however, did not address whether or how nutrient sensing by adipocytes influences the dietary response of GSCs and their descendants.

Drosophila adipocytes, together with hepatocyte-like oenocytes, compose the fat body (Figure 5.1A), a nutrient-sensing organ with endocrine roles (Arrese and Soulages 2010, Colombani et al. 2003, Rajan and Perrimon 2012). In the larval fat body, TOR activation

downstream of amino acid sensing results in the production of unknown factors that modulate overall growth of the organism (Colombani et al. 2003). In both the larval and adult fat body, sensing of sugars and lipids leads to the production of a leptin-like cytokine, Unpaired 2 (Upd2), which controls the secretion of brain insulin-like peptides (Rajan and Perrimon 2012). Here, we report that partially inhibiting amino acid transport in adult adipocytes results in a specific reduction in the number of ovarian GSCs and that, surprisingly, this effect is independent of TOR signaling. Instead, reduced amino acid levels and the consequent increase in uncoupled tRNAs trigger activation of the GCN2-dependent amino acid response pathway within adipocytes, causing increased rates of GSC loss. These results indicate that amino acid sensing by adipocytes through a TOR-independent mechanism is communicated to GSCs to control their maintenance, thereby contributing to their response to diet. Our findings bring to light the importance of elucidating how adipocytes contribute to the regulation of various adult stem cell types by diet, and how these mechanisms might be adversely affected in obese individuals.

Materials and Methods

Drosophila strains and culture conditions

Fly stocks were maintained at 22-25°C on standard medium containing cornmeal, molasses, yeast, and agar. Standard medium supplemented with wet yeast paste was used for all experiments, except for Figure 5.1F, where flies were kept on molasses/agar plates with no yeast. Previously described fat body Gal4 lines were used: *Adh-Gal4* (Fischer et al. 1988), *cg-Gal4* (Rusten et al. 2004), *FB-Gal4* (Grönke et al. 2003), *r4-Gal4* (DiAngelo et al. 2009),

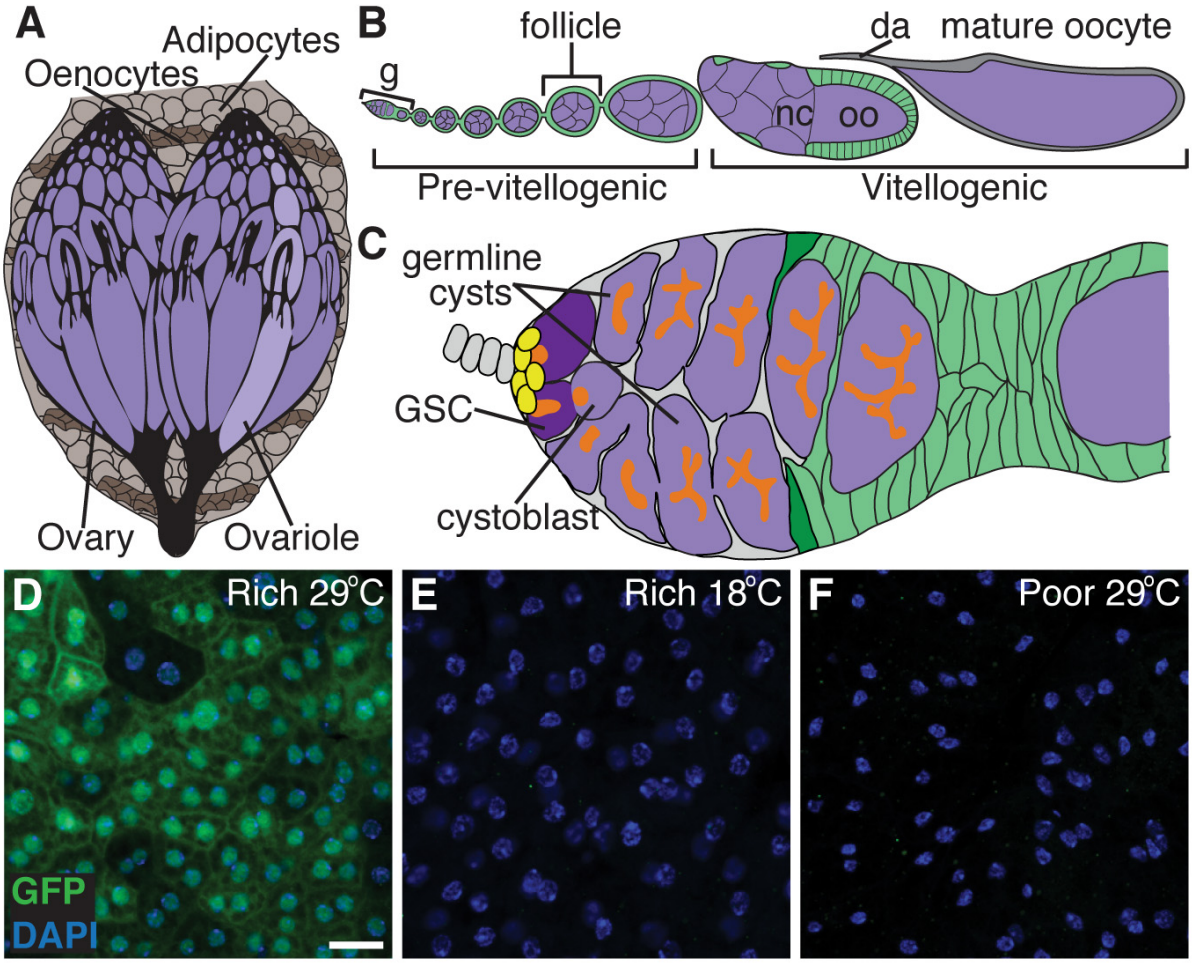


Figure 5.1. A tool to determine how genetic manipulation of nutrient-dependent pathways in adult adipocytes impacts the GSC lineage in the *Drosophila* ovary. (A) The *Drosophila* fat body is an endocrine organ awash in hemolymph, and composed of sheets of adipocytes intercalated with hepatocyte-like oenocytes. The fat body underlies the cuticle and surrounds the brain, gut and ovaries in females. **(B)** Developing follicles arranged in chronological order make up an ovariole. Follicles, formed in an anterior germarium (g), are germline cysts (one oocyte, oo, plus 15 nurse cells, nc; purple) surrounded by follicle cells (green), and develop to form a mature egg containing a dorsal appendage (da). (**C**) Each germarium contains two or three GSCs in a well-defined niche composed primarily of cap cells (yellow), and each GSC division yields a GSC and a cystoblast that forms a 16-cell cyst. GSCs and other early germline stages are identifiable based on the position and morphology of a germline-specific organelle, the fusome (orange). Follicle cells derived from follicle stem cells (dark green) envelop the cyst, making a follicle. (**D-F**) In females raised at 18°C and subsequently switched to 29°C, *Gal80^{ts}*; *Lsp2* drives *UAS* transgene expression specifically in adult adipocytes (see Figures 5.2 and 5.3). A *UAS-GFP* reporter (green) driven by *Gal80^{ts}; Lsp2* shows robust expression in adipocytes on a rich diet at 29°C (D), but is not expressed either at 18°C (E) or on a poor diet (F). DAPI (blue) labels nuclei. Scale bar, 50 μm.

pumpless-Gal4 (Colombani et al. 2003), and *3.1Lsp2-Gal4* (Lazareva et al. 2007) . The temperature-sensitive *tub-Gal80^{ts}* transgene has been described (McGuire et al. 2003). *UAS-RNAi* lines obtained from the Vienna *Drosophila* RNAi stock center (http://stockcenter.vdrc.at), and the Transgenic RNAi Project (http://www.flyrnai.org) collection at Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu) for knockdown of amino acid transporters are listed in Table 5.1. Other *UAS-RNAi* lines used were: *P{GD14098}v42184* (line 1) and *P{GD14098}v42185* (line 2), against *Aats-arg*; *P{KK102374}VIE-260B*, against *Aats-his*; *P{TRiP.HMS00763}attP2*, against *Aats-lys*; *P{TRiP.GL00267}attP2*, against *Gcn2*; *P{UAS-GFP.dsRNA.R}143*, against *GFP* (used as a control); *P{TRiP.JF01545}attP2*, against *white* (used as a control). The *UAS-CG12773^{RNAi}; UAS-Gcn2^{RNAi}* and *tub-Gal80^{ts}; 3.1Lsp2-Gal4* (*Gal80^{ts}; Lsp2*) lines were generated by standard crosses. The following *UAS* lines have been described: *UAS-slif* antisense (Colombani et al. 2003), *UAS-Tsc1; UAS-Tsc2* (Tapon et al. 2001), and *UAS-RagA^{T16N}* (Kim et al. 2008). Other genetic elements used are described in FlyBase (http://www.flybase.org).

Adult adipocyte-specific genetic manipulations

For adult adipocyte-specific genetic manipulation, females of genotypes *yw; tubP-Gal80^{ts}/+; 3.1Lsp2-Gal4/UAS-X* or *yw; tubP-Gal80^{ts}/UAS-X; 3.1Lsp2-Gal4/+* were used. (*UAS-X* represents any of the *UAS* transgenes in this study.) Females were raised at 18^{o}C, the permissive temperature for Gal80^{ts}, to keep transgene expression off during development. Newly eclosed females were maintained at 18^{o}C for three days and then switched to 29^{o}C, the restrictive temperature for Gal80^{ts}, for various lengths of time to induce transgene expression prior to dissection and/or analyses.

Immunostaining and fluorescence microscopy

All tissues were dissected in Grace's medium (BioWhittaker) and fixed in 5.3% formaldehyde (Ted Pella) in Grace's medium at room temperature for the following amounts of time: 13 minutes for ovaries, 20 minutes for abdominal carcasses (containing attached fat body) or brains, and 1 hour for guts. Tissues were rinsed and washed three times in 0.1% Triton X-100 (Sigma) in phosphate-buffered saline (PBS), or PBT, and subsequently blocked in 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (NGS; Jackson ImmunoResearch) in PBT, or blocking solution, for three hours at room temperature or overnight at 4°C. Tissues were incubated overnight at 4°C in the following primary antibodies diluted in blocking solution: rabbit anti-GFP (Torrey Pines, 1:2,500); mouse monoclonal anti-Hts (1B1) (DSHB; 1:10); mouse anti-a-spectrin (DSHB; 1:50); mouse monoclonal anti-Lamin C (LC28.26) (DSHB; 1:100); rat monoclonal anti-E-cadherin (DCAD2) (DSHB, 1:100); rabbit anti-pMad (Smad 3, #1880) (Epitomics; 1:100). [Please note that this particular Smad 3 antibody is widely used in *Drosophila* to specifically detect pMad (Ables and Drummond-Barbosa 2010, Hayashi et al. 2009, Issigonis and Matunis 2012, Ma et al. 2014, Matsuoka et al. 2013, Sulkowski et al. 2014).] Tissues were washed in PBT, and incubated for two hours at room temperature in 1:200 AlexFluor 488- or 568-conjugated secondary antibodies (Molecular Probes). Samples were washed, and ovaries, brains, guts, and fat bodies (scraped off from carcasses) were mounted in Vectashield containing DAPI (Vector Labs). For visualization of lipid droplets, fixed and blocked carcasses were incubated in 1:200 Nile Red (Sigma) in 50% glycerol in PBS for 10 minutes at room temperature. Fat bodies were mounted in 90% glycerol in PBS containing 0.5 mg/ml DAPI (Sigma). Data were collected with a Zeiss AxioImager-A2 fluorescence microscope, or a Zeiss LSM700

Table 5.1. Amino acid transporters tested in this study.

AAT[a]	Type[b]	RNAi transgene #1	RNAi transgene #2[c]	Fat body expression[d]
CG1607[e]	polyamine transporter	*P{GD4651}v14925*	*P{KK107364}VIE-260B*	larval/adult
CG1628	L-ornithine transporter	*P{KK108506}VIE-260B*	*P{GD8885}v47475*	adult
CG4991	n.s.[f]	*P{GD3406}v30263*	-	-
CG5535	cationic amino acid transporter	*P{KK100907}VIE-260B*	-	-
CG7255	cationic amino acid transporter	*P{KK110010}VIE-260B*	-	-
CG7708	proline:sodium symporter; choline transporter	*P{KK109385}VIE-260B*	*P{GD3648}v30302*	-
CG7888	n.s.	*P{GD2411}v37263*	-	-
CG8785	n.s.	*P{GD1961}v4650*	-	-
CG9413	polyamine transporter	*P{KK101306}VIE-260B*	-	-
CG12531	polyamine transporter; cationic amino acid transporter	*P{KK109373}VIE-260B*	-	-
CG12773	sodium:potassium: chloride symporter	*P{KK102472}VIE-260B*	*P{GD3189}v9899*	larval/adult
CG12943	n.s.	*P{KK112469}VIE-260B*	-	-
CG13248	polyamine transporter; cationic amino acid transporter	*P{KK103406}VIE-260B*	-	-
CG13384	n.s.	*P{KK102447}VIE-260B*	*P{GD1007}v44246*	adult
CG13646	n.s.	*P{GD257}v1571*	-	-
CG13743	n.s.	*P{GD3488}v40974*	-	-
CG16700	GABA:hydrogen symporter	*P{GD3405}v45188*	-	-
CG17119	L-cystine transporter	*P{GD3122}v51127*	-	-
CG30394	n.s.	*P{GD2127}v3470*	-	-
CG32079	n.s.	*P{KK107121}VIE-260B*	-	-
dmGlut	glutamate transporter	*P{TRiP.HMS01615}attP2*	-	larval
kazachoc	potassium:chloride symporter activity	*P{TRiP.HMS01058}attP2*	-	-
minidiscs	polyamine transporter; leucine import	*P{GD453}v42485*	-	adult[g]
Ncc69	sodium:potassium:chloride symporter	*P{KK108763}VIE-260B*	-	-
pathetic	n.s.	*P{KK104735}VIE-260B*	-	larval
slimfast	polyamine transporter; cationic amino acid transporter	*slif antisense*[h]	*P{GD12619}v45590*	larval[h]

[a] AAT, amino acid transporter. The *Drosophila* genome encodes 40 predicted amino acid transporters; for 26 of them, RNAi lines were available (www.flybase.org).
[b] Type of amino acid transporter according to FlyBase annotation (www.flybase.org).
[c] The second set of RNAi lines target sequences that are different from those targeted by the first set (stockcenter.vdrc.at).
[d] Fat body expression is listed as reported in FlyBase, except where indicated.
[e] The red font indicates amino acid transporters followed up on in this study.
[f] n.s., not specified.
[g] Adult fat body expression of *minidiscs* reported in Martin et al. (2000).
[h] Larval fat body expression of *slif* and *UAS-slif* antisense transgene described in Colombani et al. (2003)

confocal microscope. For nuclear pMad quantification, the densitometric mean of individual GSC nuclei was measured from optical sections containing the largest nuclear diameter (visualized by DAPI) using AxioVision. (Please note that to achieve as much consistency as possible among samples for pMad measurements, ovaries were dissected, fixed and stained in parallel under identical conditions, and the image acquisition settings were exactly the same for all images used for quantification.)

GSC and cap cell analyses

Cap cells were identified based on their ovoid shape and Lamin C staining, and GSCs were identified based on their juxtaposition to cap cells and fusome morphology and position, as described (Hsu and Drummond-Barbosa 2009, Hsu et al. 2008). For statistical analysis of differences in GSC loss over time we used two-way ANOVA with interaction (www.graphpad.com), which, simply stated, calculates the significance of any differences measured among genotypes in how much GSC numbers change over time.

Egg counts and ovulation analyses

To measure egg production, five pairs of flies (females of appropriate genotype and *y w* wild-type males) were maintained in plastic bottles containing molasses/agar plates covered by a thin layer of wet yeast paste, in triplicate, at 29^{o}C. Plates were replaced daily, and eggs laid within the preceding 24 hours were counted on specific days throughout experiments.

For ovulation analyses, females were dissected in Grace's medium and intact ovaries were examined under a Zeiss Stemi 2000 stereomicroscope. Each ovariole in a wild-type ovary typically contains zero or one mature oocyte, recognizable by its fully developed dorsal appendage (Spradling 1993). Ovaries in which at least one ovariole contained two or

more mature oocytes were classified as having partially blocked ovulation. Images of whole ovaries were captured using a Nikon Coolpix L620 digital camera.

EdU incorporation, apoptosis assay, and quantification of vitellogenesis defects

For EdU analysis, intact ovaries were incubated for 1 hour at room temperature in 100 μM EdU (Molecular Probes) diluted in Grace's medium, washed, fixed as described, and permeabilized for 20 minutes in 0.5% Triton X-100 in PBS. Following primary antibody incubation, EdU samples were subjected to the Click-iT reaction according to the manufacturer's protocol (Life Technologies) for 30 minutes at room temperature. GSC proliferation rates were determined by calculating the fraction of EdU-positive GSCs as a percentage of the total number of GSCs analyzed per genotype. To measure follicle cell proliferation, single confocal planes transecting follicle monolayers (i.e. follicle cell fields) at the top and bottom of flatly mounted ovarioles were acquired, and the average percentage of EdU-positive follicle cells per follicle cell field was calculated. This analysis included follicle cells covering follicle stages 4 to 6, prior to the mitotic-to-endoreplication switch, as described (LaFever et al. 2010).

ApopTag Direct In Situ Apoptosis Detection Kit (Millipore) was used as described (Drummond-Barbosa and Spradling 2001). Progression through vitellogenesis was assessed using DAPI staining (Spradling 1993). Ovarioles containing vitellogenic follicles were easily distinguished from those with blocked vitellogenesis, which contained at least one dying vitellogenic follicle. Dying vitellogenic follicles were recognizable by their position within the ovariole and by the presence of pyknotic nuclei.

RT-PCR analyses

Fat bodies from two to 10 females per genotype after 10 days of RNAi induction were hand dissected in RNAlater solution (Ambion). RNA was extracted using the RNAqueous-4PCR DNA-free RNA Isolation for RT-PCR kit (Ambion) and cDNA was synthesized using the SSRII kit (Ambion) according to the manufacturer's protocols. For each primer pair, PCR was performed on both the control and corresponding RNAi samples. The primers used are listed in Table 5.2. *Rp49* primers were used as a control. Band intensity was quantified using AxioVision by subtracting background pixels from band pixels in a fixed size box (net band intensity) and normalized to the net band intensity of the corresponding *Rp49* band. Controls were set to one and experimental sample intensities were determined relative to control.

Results

A tool for specific genetic manipulation of adult adipocytes

As a first step towards specific genetic manipulation of adult adipocytes using the *UAS/Gal4/Gal80* system (del Valle Rodriguez et al. 2012), we sought to identify a Gal4 driver that, in adults, shows expression exclusively in adipocytes. We tested several Gal4 drivers with previously reported expression in the larval and/or adult fat body (Colombani et al. 2003, DiAngelo et al. 2009, Fischer et al. 1988, Grönke et al. 2003, Lazareva et al. 2007, Rusten et al. 2004) using a *UAS-GFP* reporter. Most of these fat body drivers showed expression in at least one additional adult tissue on a yeast-rich diet (Figures 5.2 and 5.3). In contrast, the *3.1Lsp2-Gal4* driver (Lazareva et al. 2007) drove robust GFP levels in adipocytes, with no detectable expression in oenocytes, brain, gut, or ovaries (Figure 5.2 and 5.3). Further, by combining *3.1Lsp2-Gal4* with a temperature sensitive *tub-Gal80^{ts}* transgene (McGuire et al. 2003) (*Gal80^{ts}; Lsp2*), we could temporally restrict its expression (Figure 5.1E,F). In females raised at the permissive temperature (18^{o}C), the active Gal80^{ts} protein

inhibits Gal4 function and prevents GFP transgene expression (Figure 5.1E). At the restrictive temperature (29°C), the Gal80^{ts} protein is inactive, allowing GFP expression exclusively in adult stages (Figure 5.1D). Expression of *3.1Lsp2-Gal4*, however, was drastically decreased on a yeast-free diet (Figure 5.1F), precluding the use of this driver under those conditions. Thus, *Gal80^{ts}; Lsp2* can be used as a tool to drive the expression of *UAS* transgenes specifically in adult adipocytes without interfering with development.

Decreased amino acid transport in adult adipocytes inhibits egg production

Amino acids are key dietary components that have systemic effects on organismal growth during development through their action in the larval fat body (Colombani et al. 2003). To test if amino acid sensing in adipocytes might have an effect on the adult GSC lineage, we knocked down individual amino acid transporters in adult adipocytes using *Gal80^{ts}; Lsp2* and available *UAS-RNAi* lines (Table 5.1, Figure 5.4A). The *Drosophila* genome encodes 40 predicted amino acid transporters (www.flybase.org). Knockdown of single transporters in adult adipocytes did not lead to gross abnormalities in ovarian follicle development, fat body morphology, or overall female health (Figure 5.4B). For approximately one third of amino acid transporters tested, however, adipocyte-specific knockdown resulted in a significant decrease in number of eggs laid (Figure 5.5). These results suggest that incomplete loss of function of single transporters (and presumably relatively small changes in intracellular amino acid levels) within adipocytes are sufficient to influence oogenesis.

Table 5.2. Primers used for RT-PCR analysis of AAT knockdown.

Gene	Forward	Reverse
CG1607	DDB788 (5'-AGTATCGGTGTGGCTGTATTG-3')	DDB789 (5'-CTGGCAGAAGTTGTTGTGTATTT-3')
CG12773	DDB763 (5'-CATGTTAATGCCCGACAG-3')	DDB764 (5'-CATAGCTCTCGTCAGCGTC-3')
CG13384	DDB790 (5'-CTGGATCGGGAGATGATGAAAT-3')	DDB791 (5'-ACGCCACAAAGAGGAAGTAG-3')
Aats-arg	DDB796 (5'-CCGAACGATCTGCTATCCTAAA-3')	DDB797 (5'-TCTTAGCCAGCTTCCATTCC-3')
Aats-his	DDB794 (5'-CCACATCGCCAAGGTCTATC-3')	DDB795 (5'-ATCGAAGCTAACTCGCTTATCC-3')
Aats-lys	DDB792 (5'-GGCTCCTACAAGGTCATCTATC-3')	DDB793 (5'-GGTATACGCGTTGCAAATCTC-3')
Gcn2	DDB811 (5'-ACACTGGCCCTAAGCCAATC-3')	DDB812 (5'-GCCTTGCTGGTGAATATGCG-3')
Rp49	DDB137 (5'-CAGTCGGATCGATATGCTAAGC-3')	DDB138 (5'-AATCTCCTTGCGCTTCTTGG-3')

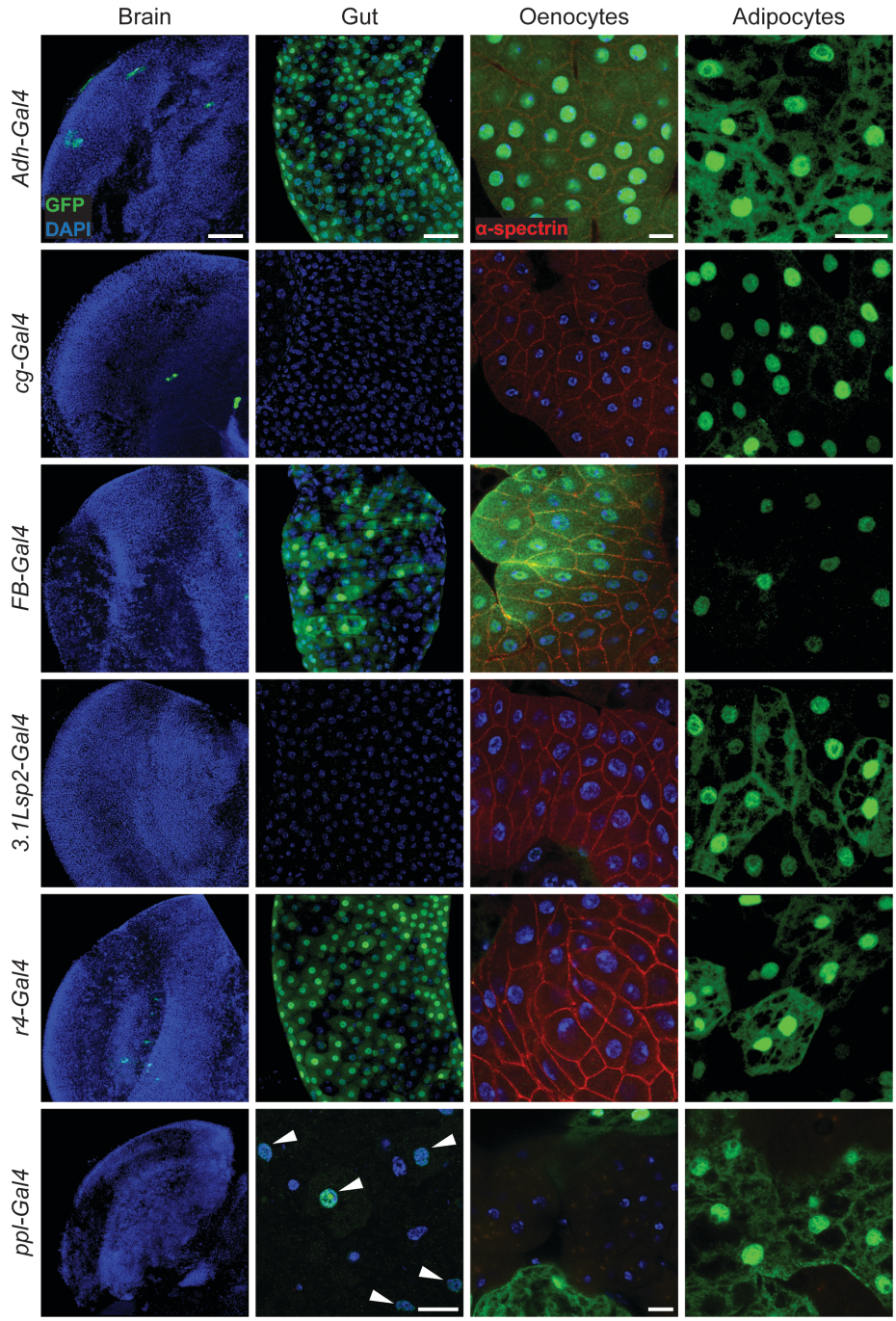


Figure 5.2. In adult females, *3.1Lsp2-Gal4* is exclusively expressed in adipocytes. Expression of *UAS-GFP* (green) induced by several larval and/or adult fat body Gal4 drivers in adult female tissues shows that only *3.1Lsp2-Gal4* is exclusively expressed in adipocytes. DAPI (blue) labels nuclei in brains, guts and oenocytes; α -spectrin (red) labels cell membranes in oenocytes (except in *ppl-Gal4*). Arrowheads indicate GFP-positive nuclei in the gut, for *ppl-Gal4*. Scale bars: 50 μm (brains), 50 μm (guts, for all except *ppl-Gal4*), 20 μm (gut,for *ppl-Gal4*), 10 μm (oenocytes), or 20 μm (adipocytes).

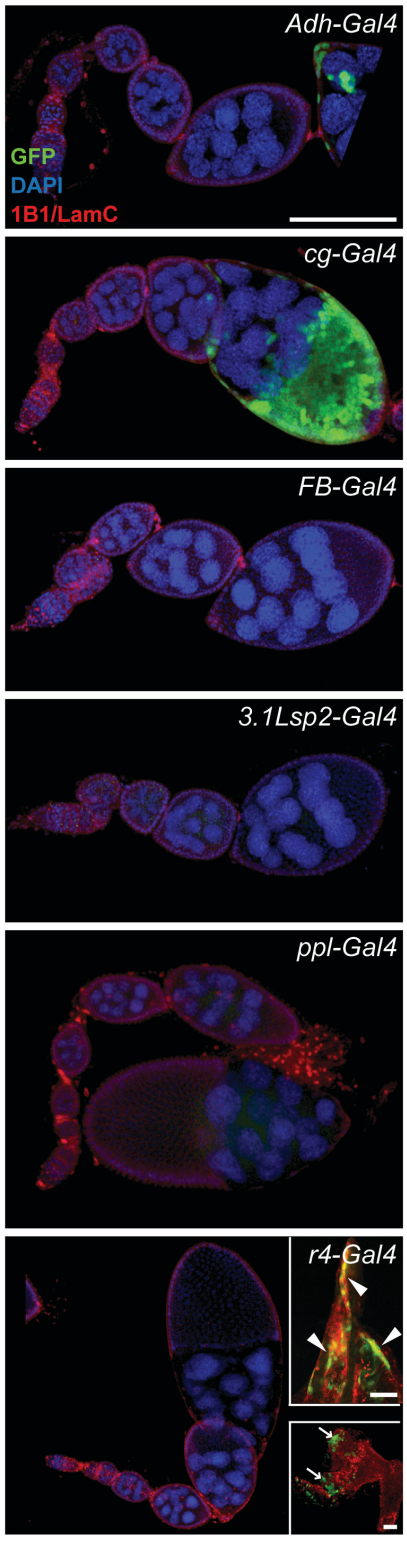


Figure 5.3. ***3.1Lsp2-Gal4*** **is not expressed in ovaries.** Analysis of *UAS-GFP* (green) induced by fat body Gal4 drivers shown in Figure 5.2 in adult ovaries shows that *3.1Lsp2-Gal4* has no ovarian expression. *Adh-Gal4* is expressed late follicle cells, including border cells (yellow arrow), *cg-Gal4* is expressed in stage 10 and later follicle cells, and *r4-Gal4* is expressed in late dorsal-anterior follicle cells (arrowheads) and oviduct (white arrows). DAPI (blue) labels nuclei; 1B1 (red) labels cell membranes; LamC (red) labels nuclear envelopes of a subset of terminally differentiated cells. Scale bars: 100 μm (main panels), 50 μm (top inset), 50 μm (bottom inset).

Reduced amino acid transport in adult adipocytes leads to increased GSC loss

Changes in GSC number or activity can contribute to alterations in egg production. We therefore determined whether GSCs are specifically affected by amino acid transport within adipocytes. Based on their pronounced egg laying reduction (Figure 5.5) and reported fat body expression (Table 5.1), we focused our analyses on females with adipocyte-specific knockdown of the amino acid transporters encoded by *CG12773*, *slimfast* (*slif*), *CG7708*, *CG1607*, *CG1628*, and *CG13384*. The number of GSCs declined significantly faster over time in females with adipocyte amino acid transporter knockdown relative to controls (Figures 5.6A, 5.7), suggesting that reductions in amino acid levels within adipocytes can be communicated to the ovary to influence GSC maintenance.

GSC loss downstream of adipocyte amino acid sensing is not a consequence of severe niche impairment or alterations in systemic insulin signaling

We next tested if decreased amino acid levels in adipocytes cause GSC loss through reduced bone morphogenetic protein (BMP) signaling from the niche, which is required for GSC maintenance (Xie and Spradling 1998). We measured the nuclear levels of pMad, a reporter of BMP signaling (Kai and Spradling 2003), and found that GSCs in adipocyte transporter knockdown females showed variable levels of pMad (Figure 5.6B,C). Specifically, there is a small (less than 50%) decrease in pMad levels for three (CG12773, CG13384, and CG1607) amino acid transporters, and an increase in those levels for the remaining transporters (CG1628, slif and CG7708). Even excluding slif and CG7708 (for which sample sizes are small) from this analysis, there is no consistent and drastic decrease in pMad levels, even though all six transporters share the same reduced GSC number phenotype. It is therefore unlikely that a severe impairment of BMP signaling is the cause of GSC loss, in agreement

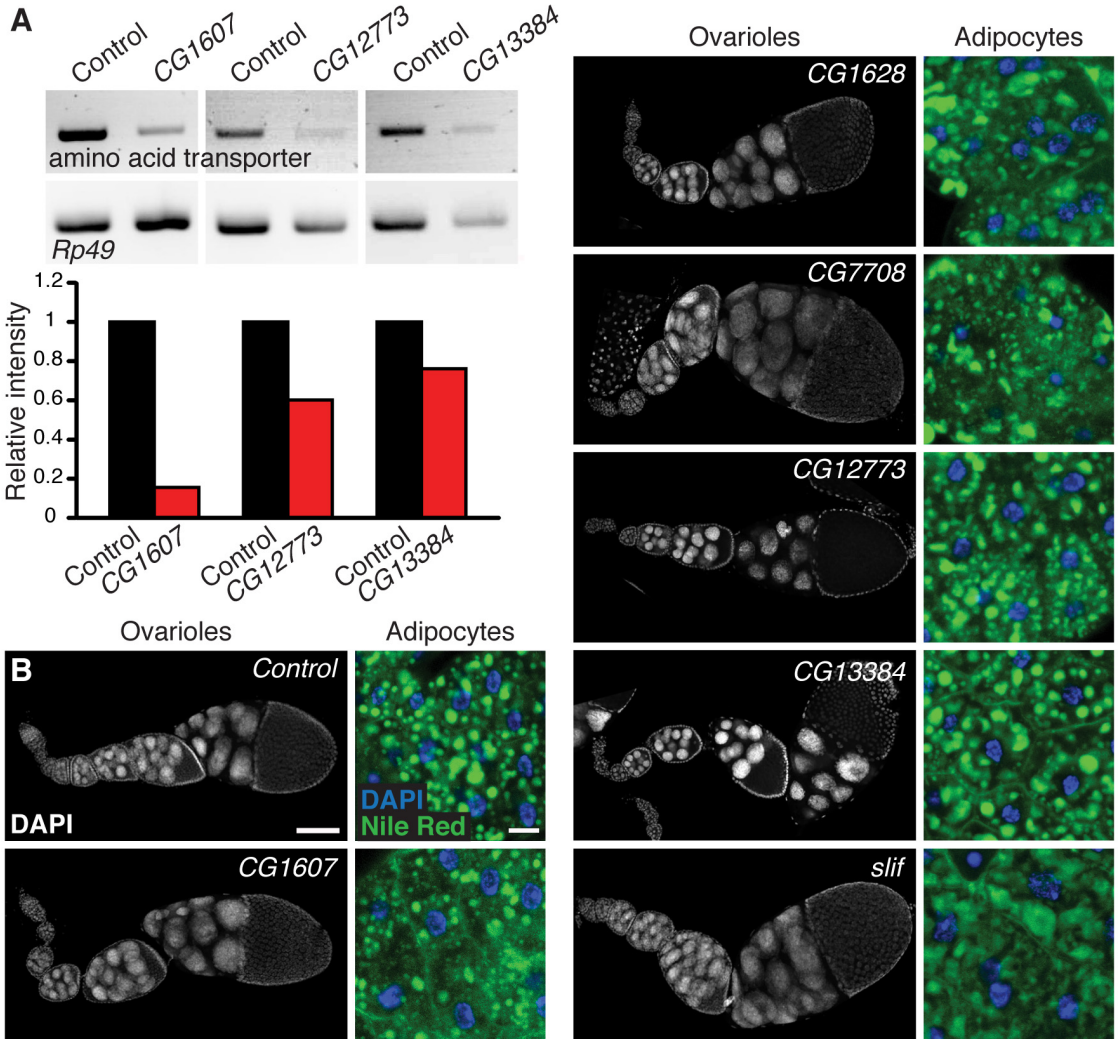


Figure 5.4. Adult adipocyte-specific knockdown of amino acid transporters does not cause obvious changes in ovarian or adipocyte morphology. (A,B) RT-PCR analysis of hand-dissected fat bodies showing knockdown of amino acid transporters (A), and normal ovariole and adipocyte morphology (B) at 10 days of *Gal80^{ts}; Lsp2*-mediated induction of RNAi or antisense transgenes against amino acid transporters or *white* control. *Rp49* is a control. DAPI (blue) labels nuclei. In adipocytes, Nile red, which fluoresces in both red and green (Greenspan et al. 1985), is shown in green. Scale bars: 100 μm (ovarioles), 10 μm (adipocytes). (See also Table 5.2.)

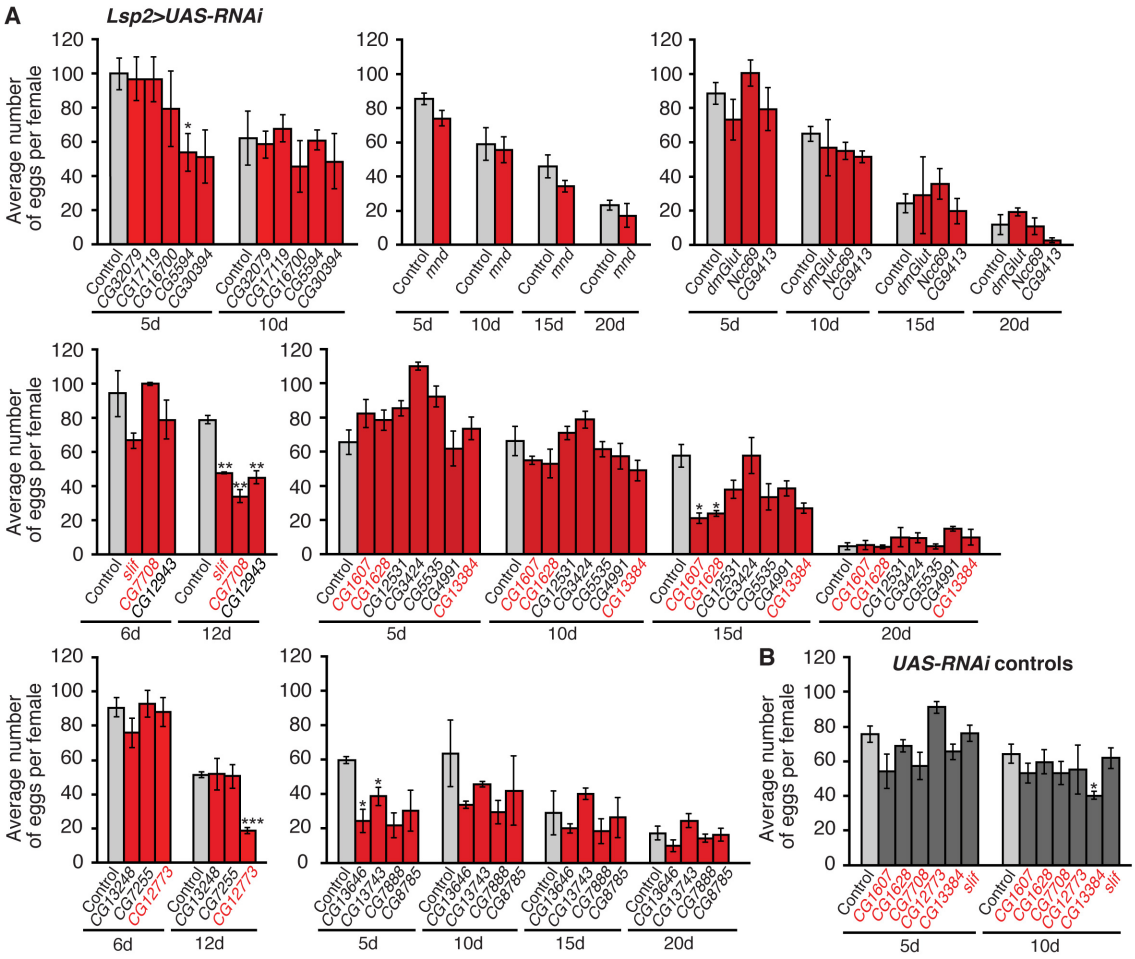


Figure 5.5. Adult adipocyte-specific knockdown of a subset of amino acid transporters encoded by *slif*, *CG7708*, *CG1607*, *CG13384*, *CG1628*, and *CG12773* results in reduced egg production. (A) Females carrying *Gal80^{ts}; Lsp2* and *UAS-RNAi* transgenes against amino acid transporters (or a *UAS-antisense* transgene (Colombani et al. 2003) in the case of *slif*) or a control *UAS-RNAi* (against *white*, an eye color gene) raised at 18°C and switched to 29°C for adult adipocyte-specific knockdown for the indicated number of days. Knockdown of a subset of transporters causes a significant decrease in the average number of eggs laid per female per day. Transporters in red font were followed up on in this study. **(B)** Control females carrying *UAS-RNAi* or *-antisense* transgenes against amino acid transporters in the absence of *Gal80^{ts}; Lsp2* and subjected to the same temperature regime as in (A) show egg laying rates statistically indistinguishable from those of a *UAS-GFP^{RNAi}* control, except for *UAS-CG13384^{RNAi}*. * P<0.05; ** P<0.01; *** P<0.001, Student's t test. Error bars indicate mean ± s.e.m. (See also Table 5.1, Figure 5.10.)

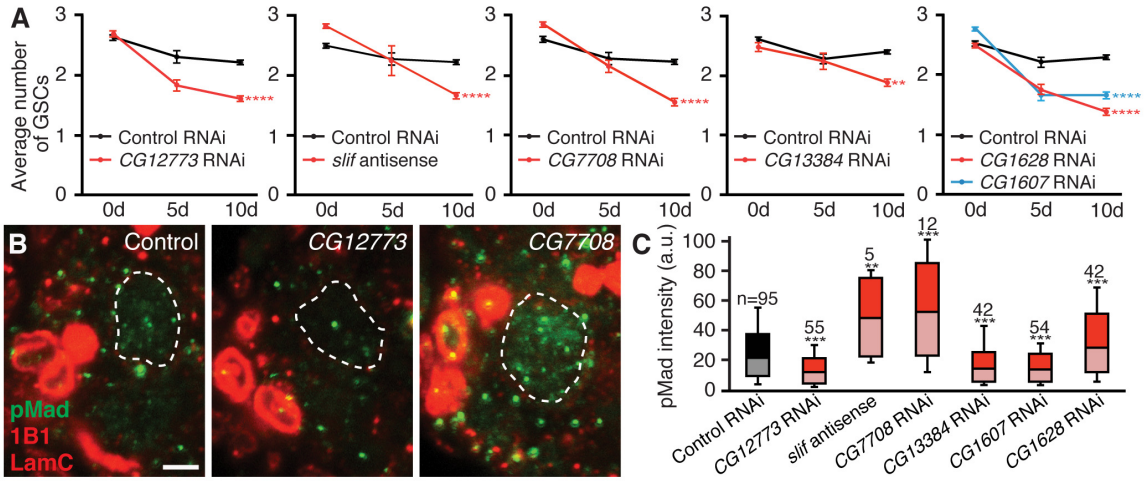
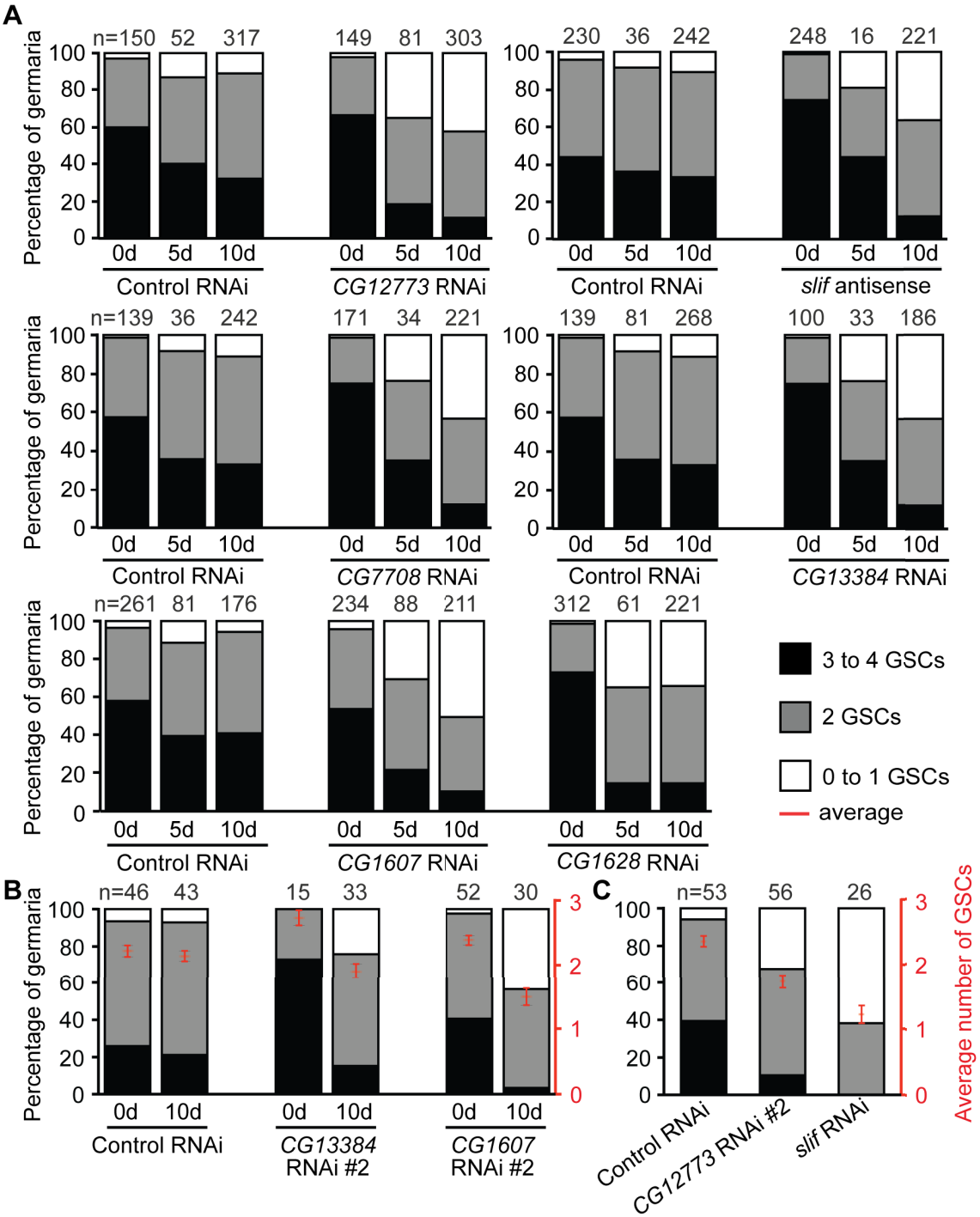


Figure 5.6. Adult adipocyte-specific knockdown of amino acid transporters leads to increased rates of GSC loss in the ovary. (A) Average number of GSCs per germarium at zero, five or 10 days of *Gal80^{ts}; Lsp2*-mediated induction of RNAi or antisense transgenes against amino acid transporters or *white* control. See Figure 5.7 for sample sizes and distribution. ***P< 0.001; ****P<0.0001, Two-way ANOVA with interaction. Error bars indicate mean ± s.e.m. **(B)** Germaria at 10 days of adipocyte-specific *GFP* control or amino acid transporter RNAi and labeled for phosphorylated Mad (pMad; green), 1B1 (red, fusome), and Lamin C (red, cap cell nuclear envelope). GSC nuclei are outlined. Scale bar, 2.5 mm. **(C)** Box-and-whisker plot of mean nuclear pMad intensity for experiment in (B). Sample sizes are included above. **P<0.01; ***P<0.001, Student's t test. (See also Figure 5.9)

Figure 5.7. Reduced amino acid transport in adipocytes leads to higher rates of GSC loss in the ovary. (A to C) Frequencies of germaria containing zero-or-one, two, or three-or-four GSCs at different days after switch to 29 °C for *Gal80ts; Lsp2*-mediated induction of a *UAS-slif antisense* or *UAS-RNAi* transgenes against amino acid transporters *CG12773*, *CG7708*, *CG13384*, *CG1607*, *CG1628*, *CG12943* or *white* control. The same data used to calculate GSC number averages in Figure 5.6 are plotted in (A). In (C), data at 10 days after switch to 29°C are shown. The reduction in average GSC numbers upon adipocyte inhibition of amino acid transport (Figure 5.6) reflects an increased percentage of germaria showing zero-or-one GSC and decreased fraction retaining two or three-or-four GSCs. The right *y*-axis in (B and C) shows the average number of cap cells per germarium. Number of germaria analyzed is shown above each bar.


A
Percentage of germaria
n=150 52 317
149 81 303
230 36 242
248 16 221
0d 5d 10d
Control RNAi
CG12773 RNAi
Control RNAi
slif antisense
n=139 36 242
171 34 221
139 81 268
100 33 186
Control RNAi
CG7708 RNAi
Control RNAi
CG13384 RNAi
n=261 81 176
234 88 211
312 61 221
Control RNAi
CG1607 RNAi
CG1628 RNAi
3 to 4 GSCs
2 GSCs
0 to 1 GSCs
average
B
n=46 43
15 33
52 30
Control RNAi
CG13384 RNAi #2
CG1607 RNAi #2
C
n=53 56 26
Control RNAi
CG12773 RNAi #2
slif RNAi
Average number of GSCs

with the observation that adipocyte knockdown of *CG1628* shows a more severe GSC loss than knockdown of *CG13384*. We also did not observe dying cells adjacent to the GSC niche (Figure 5.8A), suggesting that GSCs might be lost through differentiation, despite presumably adequate levels of BMP signaling.

During larval development, amino acid sensing by the fat body modulates systemic insulin signaling (Colombani et al. 2003), and our previous work showed that insulin-like peptides control GSC maintenance through the niche by controlling cap cell numbers and E-cadherin-mediated GSC-cap cell adhesion (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, Kwak et al. 2013). We therefore asked whether the reduction in GSC number resulting from amino acid transporter knockdown in adipocytes was due to changes in cap cell number or E-cadherin levels. The number of cap cells, however, were unaltered (Figure 5.9A). Similarly, there were no obvious differences in the levels of E-cadherin at the niche-GSC junction (Figure 5.9B; number of germaria analyzed: 96 for Control; 76 for *CG1607*; 15 for *CG1628*; 31 for *CG7708*; 74 for *CG12773*; 45 for *CG13384*; 69 for *slif*), although we cannot exclude the possibility of very minor effects on E-cadherin levels based on these visual assessments. Also inconsistent with a general reduction in insulin signaling, GSC proliferation was increased, and follicle cell division rates (a proxy for rates of follicle development) were unaltered or slightly increased in most cases upon amino acid transporter knockdown in adipocytes (Figure 5.8B,C). Thus, reduced amino acid transport in adult adipocytes causes a specific decline in GSC numbers that is independent of changes in systemic insulin signaling or severe niche alterations.

General amino acid sensing in adult adipocytes does not affect vitellogenesis but appears to partially inhibit ovulation

To determine if the GSC loss observed downstream of amino acid transporter knockdown in adipocytes is accompanied by additional alterations in the GSC lineage, we examined later stages of oogenesis. Onset of vitellogenesis and ovulation are major points of control of oogenesis by diet (Drummond-Barbosa and Spradling 2001); therefore, we examined whether amino acid transport within adipocytes may also contribute to modulation of these processes. There was no increase in the percentage of ovarioles containing dying vitellogenic follicles upon adipocyte amino acid transporter knockdown, with the exception of *slif* antisense, which caused a small but significant increase in degeneration of vitellogenic follicles (Figure 5.10). These results are also consistent with normal levels of systemic insulin signaling, which are required for intact vitellogenesis (Drummond-Barbosa and Spradling 2001, Hsu et al. 2008, LaFever and Drummond-Barbosa 2005). By contrast, knockdown of several amino acid transporters resulted in a slight increase in the fraction of ovaries showing a partial block in the ovulation of mature oocytes relative to controls (Figure 5.11A,B). The partial block in ovulation, however, was a variable phenotype that did not reach statistical significance, presumably due to the mild decrease in amino acid transport expected from knockdown of individual transporters.

Adipocyte TOR signaling controls ovulation but does not mediate the effects of adipocyte amino acid sensing on GSC maintenance

The nutrient sensor TOR acts downstream of Slif within larval adipocytes to promote organismal growth (Colombani et al. 2003), prompting us to ask if adipocyte TOR signaling mediates the effects of amino acid transporters within adult adipocytes on the ovarian GSC

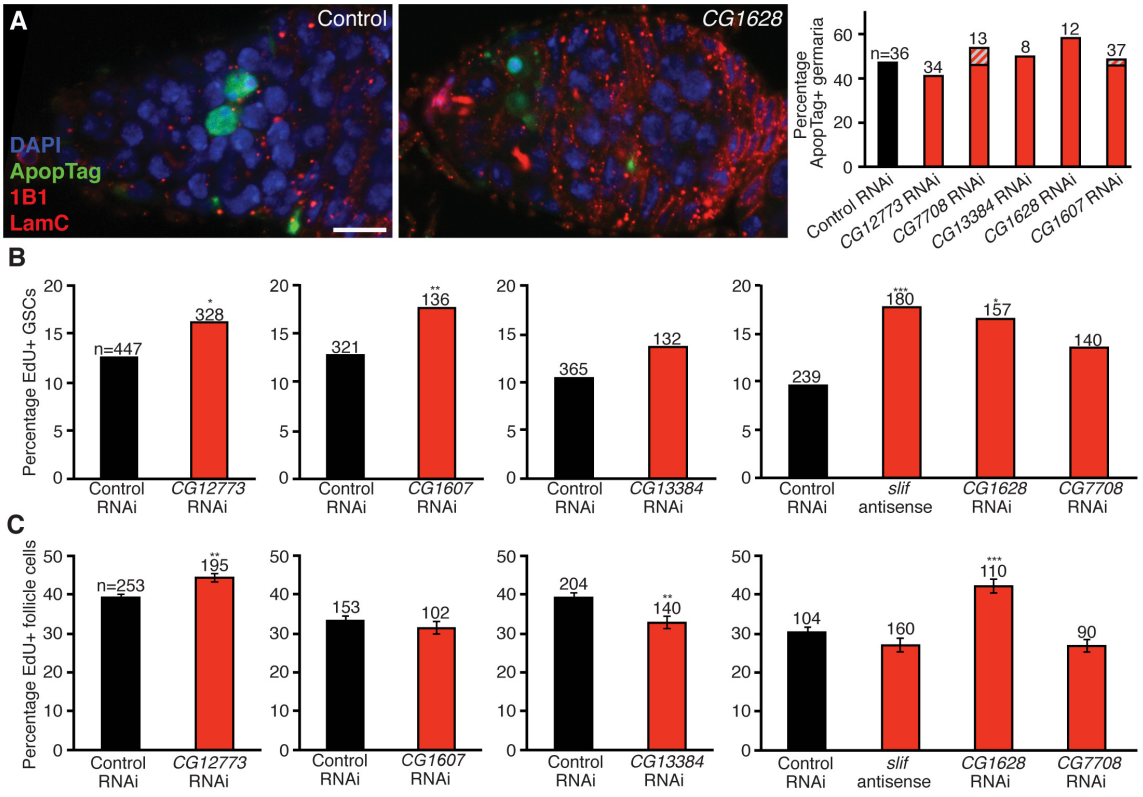


Figure 5.8. Reduced amino acid transport in adipocytes does not affect cell death within the germarium, but causes a slight increase in GSC proliferation. (**A**) Germaria from females at 10 days of adult adipocyte-specific knockdown of *CG1628* or *white* control showing some occurrence of cell death (ApopTag, green) in both cases. DAPI (blue) labels nuclei; 1B1 (red) labels fusomes; LamC (red) labels cap cell nuclear envelopes. Scale bar, 10 μm. In the graph on right, bars represent the percentage of germaria containing ApopTag-positive cells, with hatched portion indicating the fraction of those displaying ApopTag adjacent to GSC niche. (**B,C**) Frequencies of GSCs (B) or follicle cells (C) in S phase, based on EdU incorporation, at 10 days of adipocyte knockdown of amino acid transporters or *GFP* control. Number of GSCs (A) or follicle cell fields (B) analyzed is shown above each bar. **P*<0.05; ***P*<0.01; ****P*<0.001, Student's *t* test. Error bars indicate mean ± s.e.m.

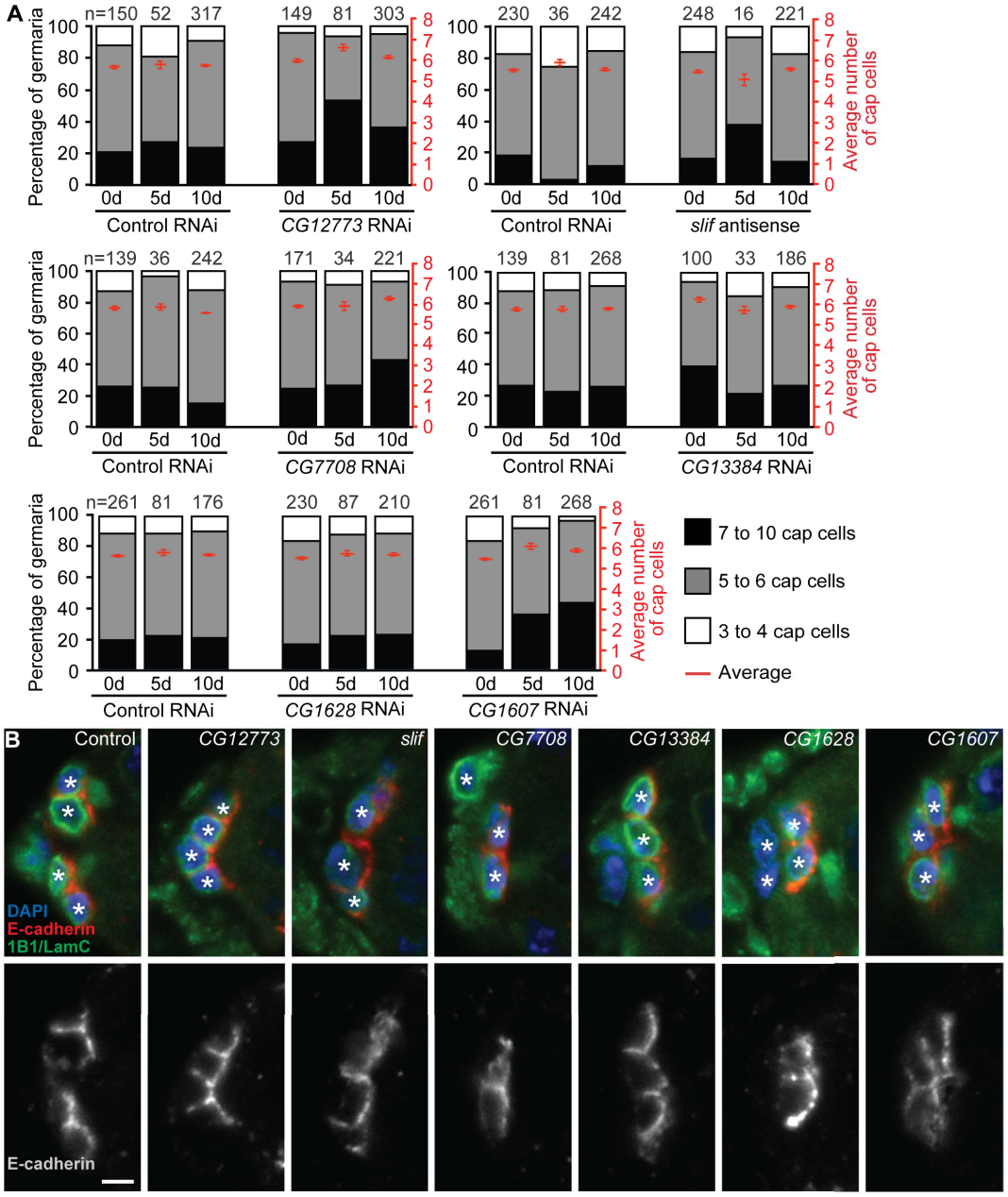


Figure 5.9. Reduced amino acid transport in adipocytes does not affect cap cell number or E-cadherin levels. (A) Frequencies of germaria containing three-or-four, five-or-six, or seven-to-10 cap cells (left *y*-axis), and average number of cap cells per germarium (right *y*-axis) at different days after switch to 29°C for *Gal80ts; Lsp2*-mediated induction of a *UAS-slif antisense* or *UAS-RNAi* transgenesagainst amino acid transporters *CG12773*, *CG7708*, *CG13384*, *CG1607*, *CG1628*, *CG12943* or*white* control. Number of germaria analyzed is shown above each bar. **(B)** Germaria from females at 10 days of adult adipocyte-specific knockdown of amino acid transporters or *white* control gene showing no obvious difference in levels of E-cadherin (red) at GSC-cap cell junctions. DAPI (blue) labels nuclei; 1B1 (green) labels fusomes; LamC (green) labels cap cell nuclear envelopes. Asterisks indicate cap cells. Scale bar, 2.5 μm.

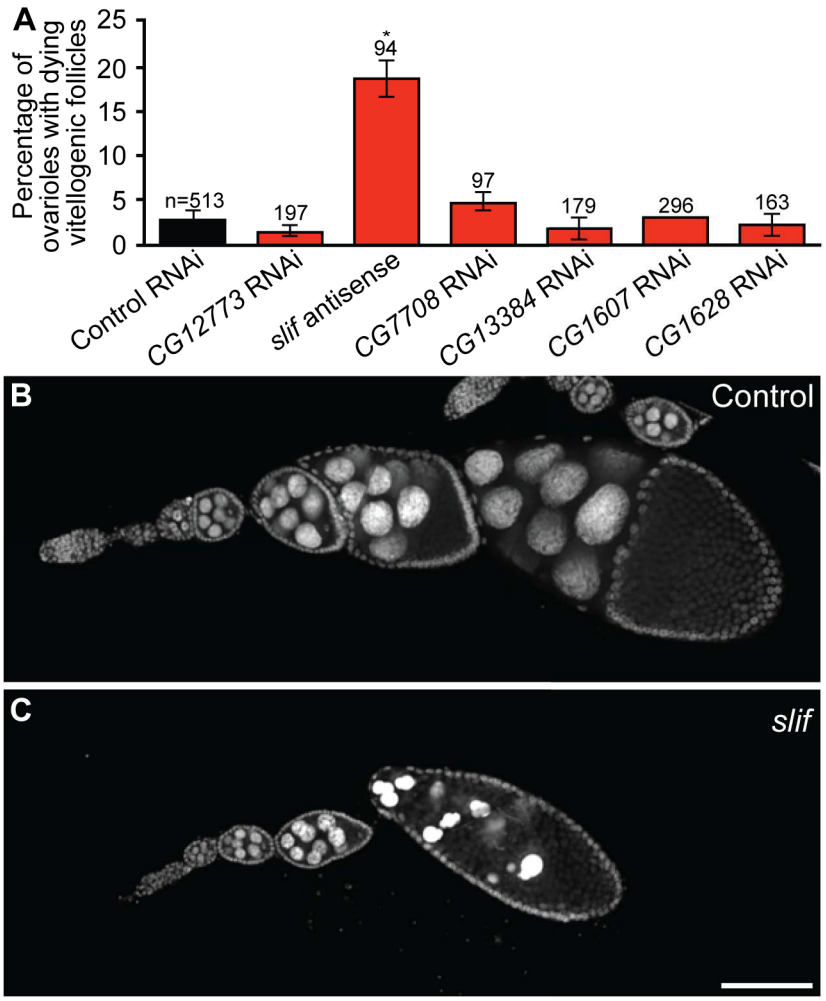


Figure 5.10. Adult adipocyte-specific knockdown of amino acid transporters does not disrupt vitellogenesis, except in the case of *slif*. (A) Percentage of ovarioles containing dying vitellogenic follicles at 10 days of adipocyte knockdown of amino acid transporters. Number of ovarioles analyzed is shown above each bar. **P*< 0.05, Student's *t* test. Error bars indicate mean + s.e.m. (**B and C)** DAPI-stained ovarioles from control (B) or *slif* (C) RNAi genotypes shown in (A). Arrow indicates degenerating follicle, recognized by the presence of pyknotic nuclei. Scale bar, 100 μm.

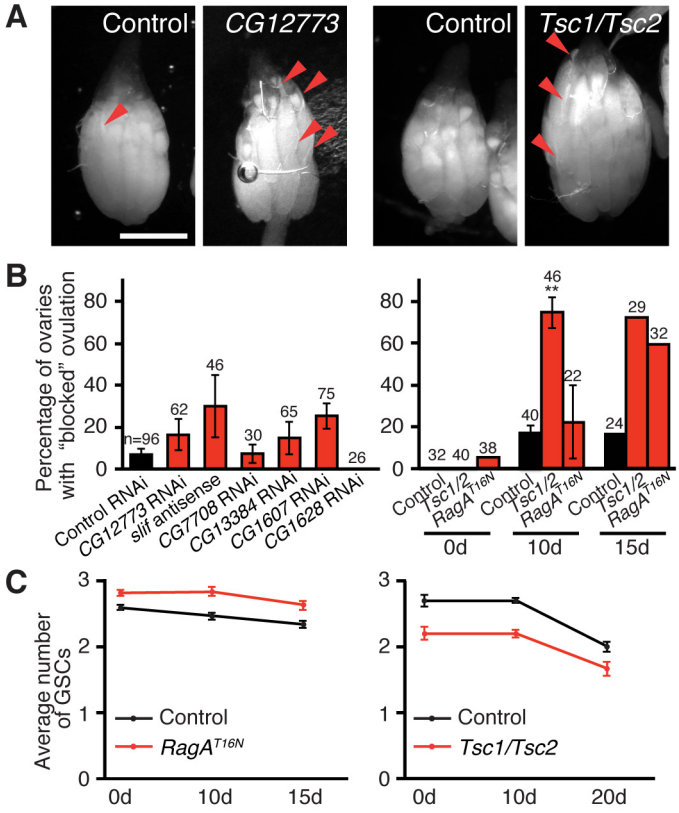


Figure 5.11. GSC loss induced by adult adipocyte- specific knockdown of amino acid transporters is independent of TOR signaling. (**A**) Ovaries at 10 days of adipocyte-specific *CG12773* knockdown or *Tsc1/Tsc2* induction showing retention of mature oocytes in subsets of ovarioles ("blocked" ovulation). Mature oocytes are recognizable by the presence of dorsal appendages (arrowheads). Scale bar, 500 μm. (**B**) Percentage of ovaries containing at least one ovariole that retains more than one mature oocyte at 10 days of adipocyte-specific amino acid transporter knockdown (left) or at different days of inhibition of TOR signaling (right). $^{**}P< 0.01$. Data from 0d and 15d time points are from one experiment, whereas 10d represents three experiments. Number of ovaries analyzed is shown above each bar. (**C**) Average number of GSCs at different days of $Gal80^{ts}$*; Lsp2*-mediated induction of a dominant-negative *RagA* ($RagA^{T16N}$) or *Tsc1/Tsc2* transgenes showing that inhibition of TOR signaling has no effect on GSC maintenance. See Figure 5.12 for sample sizes and distribution. Control is *GFP* RNAi for (A) and (B; left), and $Gal80^{ts}$*; Lsp2* alone for (B; right) and (C).

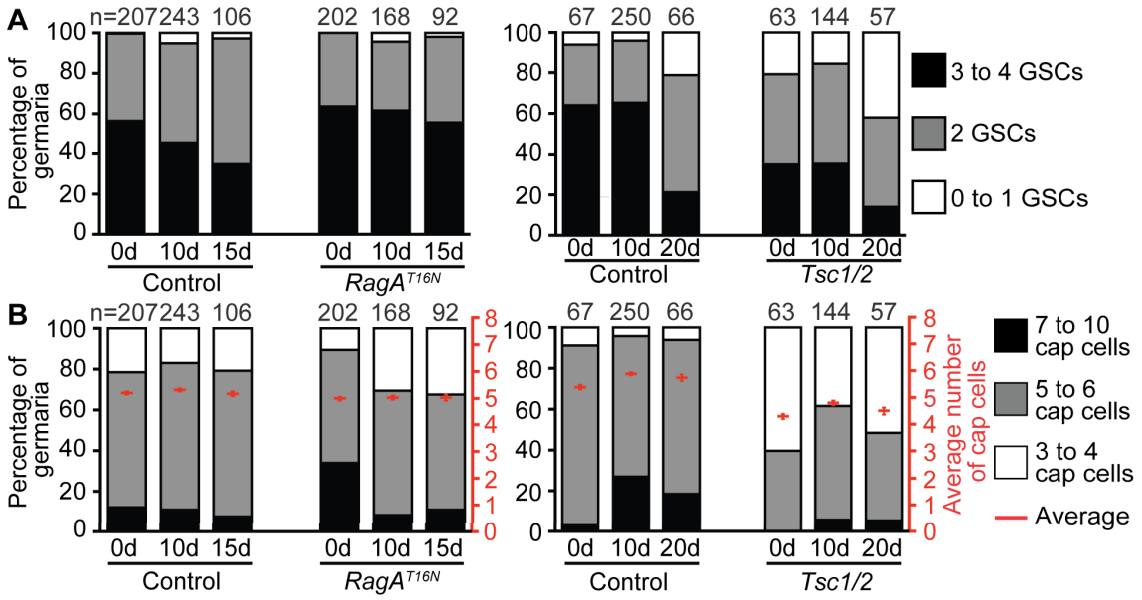


Figure 5.12. Reduced TOR signaling in adult adipoctyes does not affect GSC or cap cell number. (A and B) Frequencies of germaria containing zero-or-one, two, or three-or-four GSCs (A), or three-or-four, five-or-six, or seven-to-ten cap cells (B) at different days after switch to 29°C for *Gal80$_{ts}$; Lsp2*-mediated induction of dominant negative *UAS-RagA^{T16N}* or *UAS-Tsc1* and *UAS-Tsc2* (*Tsc1/2*) transgenes. The same data used to calculate GSC number averages in Figure 5.11 are plotted in (A). The right *y*-axis in (B) shows the average number of cap cells per germarium. Number of germaria analyzed is shown above each bar.

lineage. We inhibited TOR signaling specifically within adult adipocytes using *Gal80^{ts}; Lsp2*-driven overexpression of the Tuberous Sclerosis Complex (Tsc)1/Tsc2 complex (Tapon et al. 2001) [a negative regulator of TOR (Laplante and Sabatini 2012)], or of a dominant-negative version of RagA [RagA^{T16N} (Kim et al. 2008)], a positive regulator of TOR involved in amino acid sensing (Laplante and Sabatini 2012). Inhibition of adipocyte TOR signaling using either of these established tools caused a marked increase in the percentage of ovaries showing a partial block in ovulation relative to controls (Figure 5.11A,B). Adipocyte TOR inhibition, however, had no effect on GSC (or cap cell) numbers (Figures 5.11C, 5.12), suggesting that adipocyte amino acid levels control GSC maintenance independently of TOR signaling.

Increased levels of unloaded tRNAs and GCN2 activation in response to reduced amino acid levels in adipocytes cause GSC loss

We next hypothesized that the amino acid response (AAR) pathway may act within adipocytes to control GSC numbers. The AAR pathway, conserved from yeast to mammals, senses limitations in one or more amino acids. Reduced amino acid levels lead to an increase in unloaded tRNAs, which activate the kinase GCN2, thereby controlling downstream translational and transcriptional events (Bjordal et al. 2014, Gallinetti et al. 2013, Gietzen and Rogers 2006). Our hypothesis therefore predicts that directly increasing unloaded tRNA levels in adipocytes should reduce GSC numbers. Inhibiting or mutating aminoacyl-tRNA synthetases (the enzymes responsible for coupling amino acids to their cognate tRNAs) are well-established approaches to experimentally increase uncharged tRNA levels, thereby activating the AAR pathway under normal amino acid levels (Gallinetti et al. 2013, Gietzen and Rogers 2006). Given that Slif, a cationic amino acid transporter (Colombani et al. 2003),

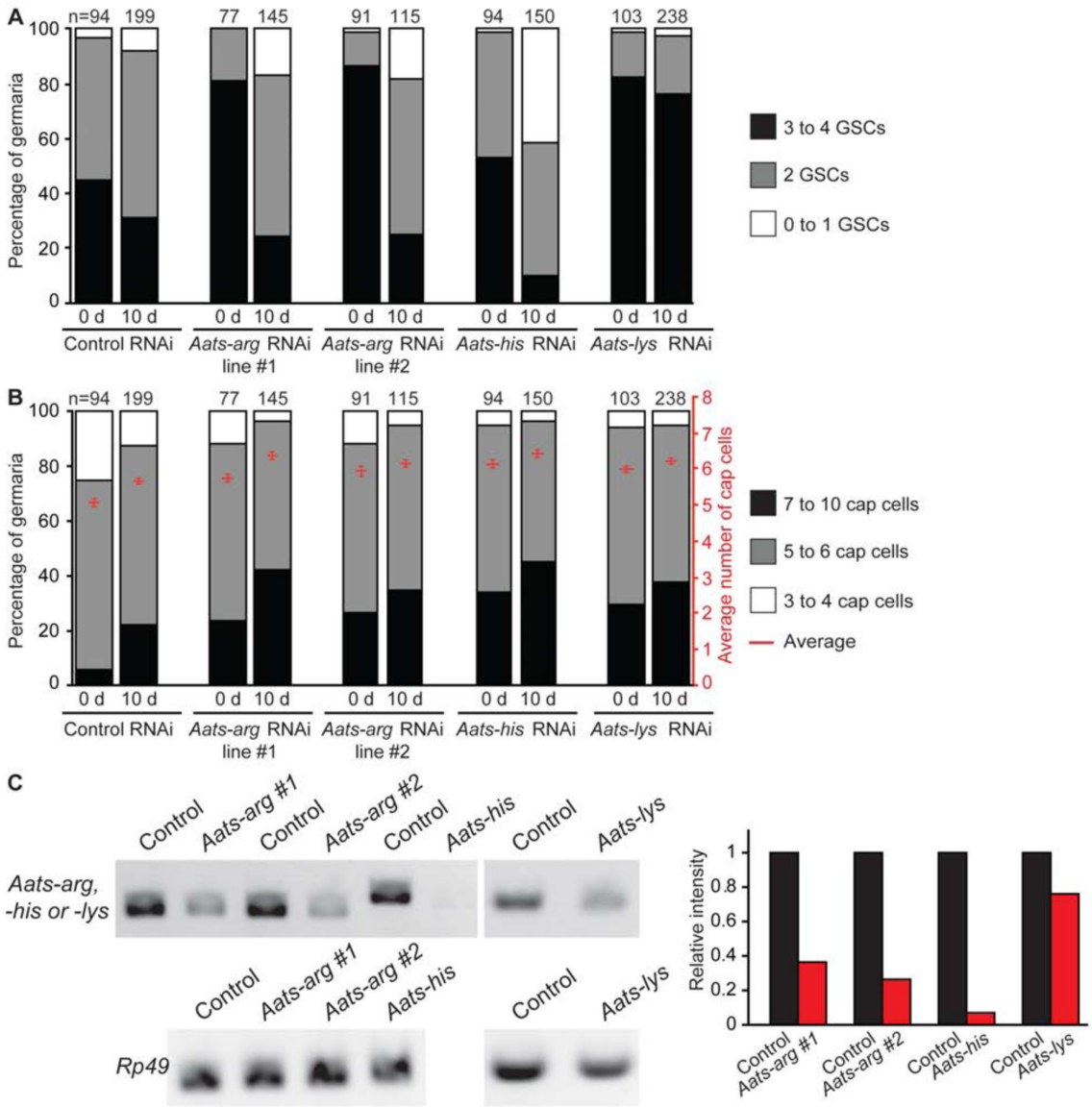


Figure 5.13. Adult adipocyte-specific knockdown of aminoacyl-tRNA synthetases causes a reduction in GSC, but not cap cell, numbers. (A and B) Frequencies of germaria containing zero-or-one, two, or three-or-four GSCs (A), or three-or-four, five-or-six, or seven-to-10 cap cells (B) at zero or 10 days after switch to 29°C for *Gal80^{ts}; Lsp2*-mediated induction of *GFP* control, *Arginyl-tRNA synthetase* (*Aats-arg*), *Histidyl tRNA synthetase* (*Aats-his*), or *Lysyl-tRNA synthetase* (*Aats-lys*) RNAi transgenes. The same data used to calculate GSC number averages in Figure 5.14A are plotted in (A). The right *y*-axis in (B) shows the average number of cap cells per germarium. Number of germaria analyzed is shown above each bar. **(C)** RT-PCR analysis of hand-dissected fat bodies showing knockdown of amino acid transporters at 10 days of *Gal80^{ts}; Lsp2*-mediated induction of RNAi transgenes against *aminoacyl-tRNA synthetases* or *GFP* control. Note that *Aats-lys* knockdown was relatively inefficient and did not alter GSC number.

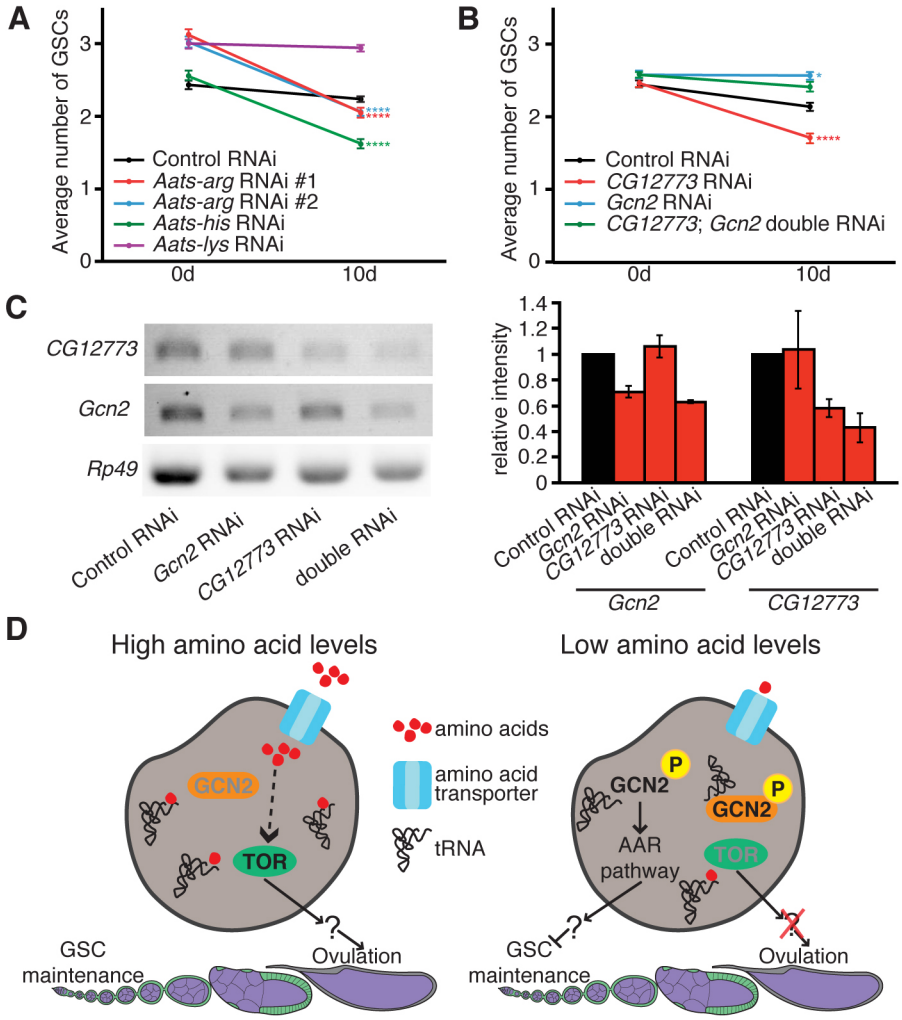


Figure 5.14. The amino acid response pathway within adipocytes contributes to the control of GSC maintenance. (**A,B**) Average number of GSCs at zero or 10 days of Gal80^{ts}; Lsp2-mediated induction of GFP control, Arginyl-tRNA synthetase (Aats-arg), Histidyl-tRNA synthetase (Aats-his), Lysyl-tRNA synthetase (Aats-lys), CG12773, Gcn2, and double CG12773 Gcn2 RNAi transgenes. See Figure 5.13 for sample sizes and distribution, and for efficiency of knockdown for experiment in (A). Numbers of germaria analyzed in (B) are: 131 for Control 0d; 71 for Control 10d; 121 for CG12773 0d; 89 for CG12773; 121 for GCN2 0d; 102 for GCN2 10d; 159 for CG12773 GCN2 double 0d; 90 for CG12773 GCN2 double 10d. * $P<0.05$; **** $P\leq0.0001$, Two-way ANOVA with interaction. Error bars indicate mean $\pm$ s.e.m. (**C**) RT-PCR analysis of hand-dissected fat bodies showing specific knockdown of Gcn2 and/or CG12773 in genotypes shown in (B). (**D**) Model for how amino acid sensing within adipocytes regulates the GSC lineage. Under high amino acid levels, the AAR pathway is off and TOR is active, resulting in optimal GSC maintenance and ovulation rates. Under lower amino acid levels, the amino acid response pathway is triggered through an increase in unloaded tRNAs and activation of GCN2 kinase, leading to GSC loss. Reduced TOR activity causes a partial block in ovulation. (See also Table 5.2.)

is among those that function in adipocytes to control GSC numbers (see Figure 5.6A), we knocked down the genes encoding Histidyl-, Arginyl-, or Lysyl-tRNA synthetases (*Aats-his*, *Aats-arg*, or *Aats-lys*, respectively) in adipocytes using *Gal80^{ts}; Lsp2* (Figure 5.13). Control or *Aats-lys* adipocyte knockdown (which was relatively inefficient; Figure 5.13) had no effect on GSC number. In contrast, knockdown of aminoacyl-tRNA synthetases using either an *Aats-his*, or two distinct *Aats-arg* RNAi transgenes in adipocytes led to a marked decrease in GSC number upon transgene induction (Figures 5.14A, 5.13A). Similarly to what we observed for amino acid transporter knockdown, there were no changes in cap cell number (Figure 5.13B). These results show that activation of the AAR pathway suffices to phenocopy the GSC loss caused by reduced amino acid transport in adipocytes. Conversely, adipocyte-specific knockdown of *Gcn2* reverts the GSC loss caused by RNAi of the amino acid transporter *CG12773* (Figure 5.14B,C), suggesting that the AAR pathway is also required to mediate the effects of adipocyte amino acid transporters on GSCs. Based on these results, we conclude that the AAR pathway within adipocytes is sufficient and required to initiate an amino acid-dependent signaling cascade of inter-organ communication to modulate GSC maintenance in the ovary.

Discussion

The specific effects of adipocyte dysfunction on normal stem cell lineages have remained largely unexplored. Yet, clear evidence shows that obesity leads to higher risk for multiple chronic diseases (Vucenik and Stains 2012). Our data support the model that amino acid levels within adipocytes are sensed through separate mechanisms that specifically affect a stem cell lineage at distinct stages (Figure 5.14D). The AAR pathway

acting within adipocytes influences maintenance of GSCs, whereas amino acid sensing through the adipocyte Rag/TOR pathway modulates the efficiency of ovulation of fully differentiated GSC daughters, or oocytes. Future studies should identify the extracellular factors acting downstream of these intra-adipocyte signaling cascades to communicate adipocyte nutritional status to the GSC lineage. This work underscores the importance of investigating the role of inter-organ communication in the control of stem cells and their differentiated daughters in a wide variety of systems. Further, it suggests that the aberrant co-option of endocrine pathways that normally tie stem cell lineages to whole body physiology might contribute to the increased cancer risk associated with obesity (Vucenik and Stains 2012).

Drosophila as model for investigating how inter-organ communication contributes to the regulation of adult stem cells

Drosophila is an ideal model for molecular physiology studies owing to the ease of cell/tissue-specific manipulations (del Valle Rodriguez et al. 2012), which are essential to dissect how individual systemic signaling events contribute to complex physiological networks. Indeed, recent years have seen an explosion in metabolism and physiology studies using *Drosophila* (Arquier et al. 2008, Colombani et al. 2012, Colombani et al. 2003, Delanoue et al. 2010, Gutierrez et al. 2007, Palanker et al. 2009, Slaidina et al. 2009, Ruaud et al. 2011, Sieber and Thummel 2009, Geminard et al. 2009). Particularly useful throughout these studies is the *UAS/Gal4* system, which allows tissue- and/or cell-type-specific genetic manipulations; however, a critical consideration when designing such studies is the specificity of Gal4 expression lest phenotypes be misinterpreted. Indeed, most of the published fat body drivers we tested were not expressed exclusively

in adipocytes in adult females. In contrast, the robust and highly specific expression of *3.1Lsp2*-Gal4 in adipocytes makes it a valuable tool for exclusive genetic manipulation of adipocytes to test how they impact not only GSCs, but also other adult stem cell types.

In addition to adipocytes, nutrient sensing by other tissues also affects GSCs. For example, insulin-like peptides secreted from the brain act directly on the germline to modulate GSC proliferation, cyst growth and vitellogenesis, and also indirectly affect GSC maintenance through effects on the niche (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, LaFever and Drummond-Barbosa 2005). Other adult stem cell types are also modulated by insulin signaling, including male GSCs and intestinal stem cells (Ables et al. 2012). Much remains unknown, however, about how other tissues influence stem cells, despite evidence suggesting endocrine roles for muscle (O'Brien et al. 2011), intestines (Reiher et al. 2011) and the brain (Nassel and Winther 2010).

Separate modes of amino acid sensing in adipocytes affect the stem cell lineage at distinct stages

Our findings that amino acid sensing by adipocytes controls GSC maintenance through the AAR pathway and ovulation through TOR clearly illustrate the high degree of specificity of adipocyte-to-ovary communication. Our results also imply that relatively small fluctuations in amino acid levels (e.g. such as resulting from partial knockdown of single amino acid transporters) within adipocytes can be effectively transmitted to the ovary to modulate stem cell number. These same slight reductions in amino acid levels resulted in less significant effects on ovulation, consistent with the distinct amino acid sensing mechanisms involved. It will be very interesting to identify and study the

effectors downstream of AAR and TOR signaling that mediate these distinct effects on the GSC lineage.

Not surprisingly, inhibition of TOR signaling impacted ovulation more severely than manipulation of single amino acid transporters, in agreement with its role downstream of transporters and as an integrator of multiple inputs, including nutrients, energy status and growth factors (Dibble and Manning 2013). It is likely that additional stimuli upstream of TOR within adipocytes also regulate ovulation.

Context-specific targets of the amino acid response pathway

The AAR pathway is evolutionarily conserved from yeast to humans; however, its downstream targets are context-dependent. In yeast, for example, phosphorylation of eIF2α by activated GCN2 causes selective upregulation of translation of the transcriptional factor GCN4, which in turn induces genes involved in amino acid transport as well as amino acid biosynthesis (Natarajan et al. 2001). Translational derepression of ATF4 (the GCN4 equivalent in *Drosophila* and humans), in contrast, leads to expression of oxidative stress genes in mouse embryonic fibroblasts (Harding et al. 2003). The targets of the AAR pathway in the context of intact multicellular organisms remain largely unidentified. Nevertheless, it is reasonable to speculate that the sets of targets regulated by the AAR in different tissues and cell types may be quite different, given the diversity of processes being modulated. For example, the AAR pathway acts in the brains of *Drosophila* larvae, mice and rats to reduce intake of food sources lacking essential amino acids (Bjordal et al. 2014, Gietzen and Rogers 2006, Hao et al. 2005). Our study demonstrates a starkly different role of the AAR pathway in adipocytes in the control of GSC numbers. A fascinating question for future studies will

be to identify the subsets of targets activated in a cell-type, context-dependent manner and to investigate how the specificity of this pathway is achieved from budding yeast to *Drosophila* adipocytes to *Drosophila* and rodent neurons to achieve such differing cellular outcomes. Our studies raise the possibility that specific targets downstream of ATF4 induced in adipocytes signal to the ovary to control GSC number. Additional studies in different tissues and conditions will elucidate how much overlap exists of targets induced by the AAR pathway. Finally, it is also possible that activation of the AAR pathway in adipocytes in response to increased levels of unloaded tRNAs could alter signals from adipocytes to GSCs downstream of either global reduction in translation or of increased levels of ATF4 and its targets (Murguia and Serrano 2012).

Adipocytes, stem cells, and increased cancer risk in obese individuals

Obesity and high calorie intake are associated with increased risk of multiple cancer types, including breast, colon and prostate cancer (Bianchini et al. 2002, McMillan et al. 2006, Xue and Michels 2010). Similar to GSCs and other stem cells (Ables et al. 2012) cancers are highly responsive to nutrient-sensing pathways, and components of the insulin and TOR pathways are often misregulated in cancers (Chen 2011, Jee et al. 2005). Given the parallels between cancer cells and stem cells, investigations of the role of adipocytes in adult stem cell regulation will likely provide valuable insights into the link between obesity and cancer risk. Based on our results, we speculate that aberrant communication of fat cells regarding their nutrient sensing status to modulate the activity of cancer cells might help explain the link between diet, adiposity, and cancer.

CHAPTER VI

IDENTIFYING ADDITIONAL ROLES FOR THE FAT BODY IN *DROSOPHILA* OOGENESIS

Introduction

The goal of this dissertation has been to understand the influence of the physiological environment on *Drosophila* oogenesis, focusing on nutrient-dependent signals. My primary work focused on the ovary-intrinsic signaling downstream of nutrient sensing (Chapters III and IV). With Dr. Alissa Armstrong, I also described an adipocyte nutrient sensing mechanism that controls GSC maintenance in the adult ovary (Chapter V). What other fat body derived factors influence *Drosophila* oogenesis, and how do they signal to the germline?

Analogous to human adipose tissue, the insect fat body has been recognized in multiple contexts as a regulator of organismal physiology. For example, the fat body is the center of the immune response (Kounatidis and Ligoxygakis 2012), and there is growing evidence that immunity and metabolism are linked (Dionne 2014). Furthermore, recent studies in *Drosophila*, including our own, suggest that adipokine signaling modules are conserved in flies (Rajan and Perrimon 2012, Kwak et al. 2013, Laws et al. 2015). Since the fat body serves as both a storage organ, reflecting the nutritional history of the organism, and an endocrine organ, secreting various factors that influence peripheral tissues, and is insulin-sensitive (DiAngelo and Birnbaum 2009), it could mediate the ovarian response to diet in multiple ways. In addition to hypothetically sensing fat body nutrient reserves, the ovary could receive secreted signals from the fat

body directly or be affected by fat body-induced changes in physiology. Studies in the adult fat body to this point have been limited, but recent progress [Chapter V, (Chatterjee et al. 2014, Banerjee et al. 2012, Rajan and Perrimon 2012)] indicates that it acts as a dynamic signaling center.

The experiments described in this chapter represent unpublished efforts further toward understanding the role of the fat body in modulating the physiological environment of the fly and controlling oogenesis. It encompasses preliminary studies investigating germline non-autonomous roles for *AdipoR* and outlines approaches for determining the adiponectin-like ligand in *Drosophila*. Furthermore, it elaborates on attempts by a previous graduate student in the lab, Leesa LaFever Sampson, to establish the cellular origin of adult adipocytes in order to expand our genetic toolkit for fat body manipulation. Finally, it addresses previous observations in our lab regarding GSC activity in lipid storage mutants. While these studies are incomplete, they will serve as a point of reference for future work in our lab.

Methods

Drosophila strains and culture conditions

Flies were maintained at 23-25^{o}C on standard cornmeal/agar/molasses media unless otherwise noted. The $AdipoR^{27}$ allele was generated as described (see Chapter III). For larval ovary experiments, $AdipoR^{27}$ and a deficiency uncovering the *AdipoR* locus were balanced over actin-GFP, TM3 for selection of appropriate genotypes: $Df/AdipoR^{27}$ or sibling controls. To specifically induce *UAS-AdipoR RNAi* in adult tissues, flies carrying a *UAS*-inducible RNAi transgene (see Table 6.1) were crossed to flies carrying a

Table 6.1. RNAi lines used in AdipoR and candidate ligand knockdown studies.

Target gene	RNAi transgene(s)
w **(control)**	*P{TRiP.JF01545}attP2*
GFP **(control)**	*P{UAS-GFP.VALIUM10}attP2*
AdipoR	*P{GD3112}v40936*
CG7916	*P{GD5385}v12397*
CG33306	*P{GD1247}v3350* (line 1) *P{KK106129}v100395* (line 2)
CG8997	*P{GD5384}v45945*
CG7968	*P{GD5387}v13489*
CG7953	*P{GD5386}v30765* (line 1) *P{KK106300}v106719* (line 2)

tissue-specific driver and a temperature sensitive *tubP-Gal80^{ts}*. The temperature-sensitive *tub-Gal80^{ts}* transgene has been described (McGuire et al. 2003). Crosses were raised at 18^{o}C, the permissive temperature for Gal80^{ts}, to keep transgene expression off during development. Newly eclosed *c587-Gal4-UAS-RNAi*, *3.1Lsp2Gal4-UAS-RNAi* or *FB-Gal4-UAS-RNA*i females were fed wet yeast at 18^{o}C for two days in the presence of males (t=0) and then switched to 29^{o}C, the restrictive temperature for Gal80^{ts}, for the indicated number of days, with daily feeding, prior to dissection. Gal4-*UAS-white RNAi* or sibling controls were used as controls, as indicated.

For candidate ligand knockdown, flies carrying *UAS*-inducible RNAi transgenes (see Table 6.1) were crossed to *act5C-Gal4* flies and reared at 25^{o}C. Newly eclosed *act5C-Gal5-UAS-RNAi* females were cultured in the presence of males for 2 days at 25^{o}C with daily wet yeast feeding (t=0), and then shifted to 29^{o}C for 10 days, with daily feeding, prior to dissection.

Immunostaining and fluorescence microscopy

Adult ovaries were dissected in Grace's Insect Medium (Lonza), teased apart, and fixed for 13 min in 5.3% formaldehyde (Ted Pella) in Grace's. Samples were rinsed and washed four times in 0.1% Triton X-100 (Sigma) in phosphate-buffer saline (PBS), or PBT, and blocked for at least 3 h at room temperature or overnight at 4°C in 5% bovine serum albumin (BSA; Sigma) and 5% normal goat serum (NGS; Jackson ImmunoResearch) in PBT unless otherwise noted. Samples were incubated at 4 °C overnight with primary antibodies in blocking solution at the following concentrations: mouse anti-Hts (1B1) (DSHB; 1:10); mouse anti-Lamin C (LC28.26) (DSHB; 1:100).

After primary antibody incubation, samples were washed for 2 h in PBT and incubated for 2 to 4 h in Alexa Fluor 488- or 568-conjugated goat species-specific secondary antibodies (1:200, Invitrogen), washed for at least 1 hour, and mounted in Vectashield with DAPI (Vector Laboratories).

Larval ovaries were processed as described (Matsuoka et al. 2013). Ovaries were dissected with fat bodies into glass-bottomed dishes with EBR [130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes (pH 6.9)] and fixed in 6% formaldehyde in buffer B [16.7 mM KH_2PO_4/K_2HPO_4 (pH 6.8), 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$] for 10 minutes. Samples were rinsed, then washed four times in PBT and blocked for at least 30 minutes at room temperature in 5% NGS in PBT. Samples were incubated in a humidified chamber at 4°C overnight with primary antibodies in blocking solution at the following concentrations: mouse anti-Hts (1B1) (DSHB; 1:20); rabbit anti-vasa (gift of Dr. Phil Lasko; 1:1000). After primary antibody incubation, samples were washed for 30 minutes in PBT and blocked again for 30 minutes at room temperature in 5% NGS in PBT. Samples were stained with Alexa Fluor 488- or 568-conjugated goat species-specific secondary antibodies (1:200, Invirogen) for 3.5 hours at room temperature, washed for 30 minutes in PBT, and mounted in Vectashield with DAPI. During mounting, ovaries were separated from fat bodies, and no weight was applied to the coverslip to preserve tissue architecture.

Confocal images were acquired using a Zeiss LSM 700 microscope, analyzed using either Zeiss ZEN 2009 or Image J software, and equally and minimally enhanced via histogram using Adobe Photoshop CS4.

Adult fat body lineage labeling and X-gal staining

Crosses to generate *hsflp/+; x-15-33/x-15-29* (Harrison and Perrimon 1993) flies were set, cultured for three days, and then tossed daily onto vials with dry yeast every 24 hours for 10 consecutive days. After 10 days, the vials were simultaneously heat shocked for 1 hour at 37 ^{o}C. Flies were cultured on wet yeast for 3 days after eclosion; both fat bodies and ovaries were dissected in cold Grace's Insect Medium (Lonza). For fat body dissections, the entire abdominal cuticle was fixed and stained to prevent loss of material during processing. Samples were fixed for 8 minutes in 0.5% gluteraldehyde in Grace's Medium, then rinsed three times with phosphate buffered saline PBS containing 0.1% Tween-20 (PBS-Tween), then washed for 10 minutes in PBS-Tween with rotating. Samples were then stained with 0.8% X-gal in warm Fe/NaP solution at 37 degrees in the dark overnight, washed three times for at least 10 minutes and mounted in 90% glycerol containing 20 mg/ml *n*-propyl gallate. Samples were imaged with an Axio A.2 Imager and brightened equally and minimally in Adobe Photoshop CS4.

EdU incorporation assay

EdU incorporation was performed as described (Ables and Drummond-Barbosa 2013). Briefly, ovaries from 4-5 day old flies were dissected in Grace's medium at room temperature, and incubated in 100 mM EdU (Invitrogen) in Grace's medium for 1 h prior to being teased apart, fixed, and stained as above. EdU was detected with AlexaFluor-594 via Click-It chemistry using manufacturer's instructions (Invitrogen) following secondary antibody incubation. GSC proliferation rates were compared by calculating the fraction of EdU-positive GSCs as a percentage of the total number of GSCs analyzed per genotype.

RT-PCR analysis

To determine the expression of candidate ligands in various tissues, 10-15 *yw* females were hand-dissected in RNAlater solution (Ambion). Gut, ovary, fat body, the thorax ("muscle"), and head were separated for separate analysis, or whole flies were processed as indicated. RNA was extracted using the RNAqueous-4PCR DNA-free RNA Isolation for RT-PCR kit (Ambion) and cDNA was synthesized using the SSRII kit (Ambion) according to the manufacturer's protocols. The primers used are listed in Table 6.2. *Rp49* primers were used as a control.

Results

AdipoR does not appear to be required for larval ovary development or in the adult ovarian niche

Many studies have implicated adiponectin signaling in sensitizing tissues to insulin; in contrast, our work demonstrated that *AdipoR* is not required in the *Drosophila* germline for insulin-dependent processes (Chapter III). However, this result does not preclude the possibility of AdipoR controlling insulin sensitivity in other contexts. We reasoned that, although AdipoR does not sensitize the adult ovarian germline to insulin, it might be required in other *Drosophila* cells or developmental stages dependent on insulin signaling. ILPs, act on multiple cell types in the ovary, both during development and in the adult tissue. In the developing ovary, primordial germ cells (PGCs) proliferate clonally to establish a germline precursor pool (Zhu 2003), remaining almost completely undifferentiated through larval development (King 1970). By the late third larval instar (LL3), PGCs cease dividing and are positioned next to organized terminal filament structures and supported by a population of stromal intermingled cells (ICs, Figure 6.1A)

Table 6.1. Primers used for RT-PCR analysis of AdipoR candidate ligands

Gene	Forward	Reverse
RP49	**DDB137** 5'-CAGTCGGATCGATATGCTAAGC-3'	**DDB138** 5'-AATCTCCTTGCGCTTCTTGG-3'
CG8997	**DDB825** 5'-AGCCCATTGGTGAGTATTCCA-3'	**DDB826** 5'-GGTAAAGCCACAGGGCATCT-3'
CG7916	**DDB827** 5'-AAGCCATCCATCACCTGACC-3'	**DDB828** 5'-AGACCCTGACTATCGGGCTT-3'
CG7953	**DDB829** 5'-TGAAGGTGAGACTCGGTGAA-3'	**DDB830** 5'-AGCAATACGCAGGTTGACCA-3'
CG7968	**DDB 831** 5'-GGCTCAGATGGCCTACAAGG-3'	**DDB 832** 5'-TGCAACTGCGGTTCAGTATG-3'
CG33306	**DDB833** 5'-CCTTTGTCAGGTGAGAATATGAAG-3'	**DDB834** 5'-ATCCTTCACACGAAGCTGGG-3'

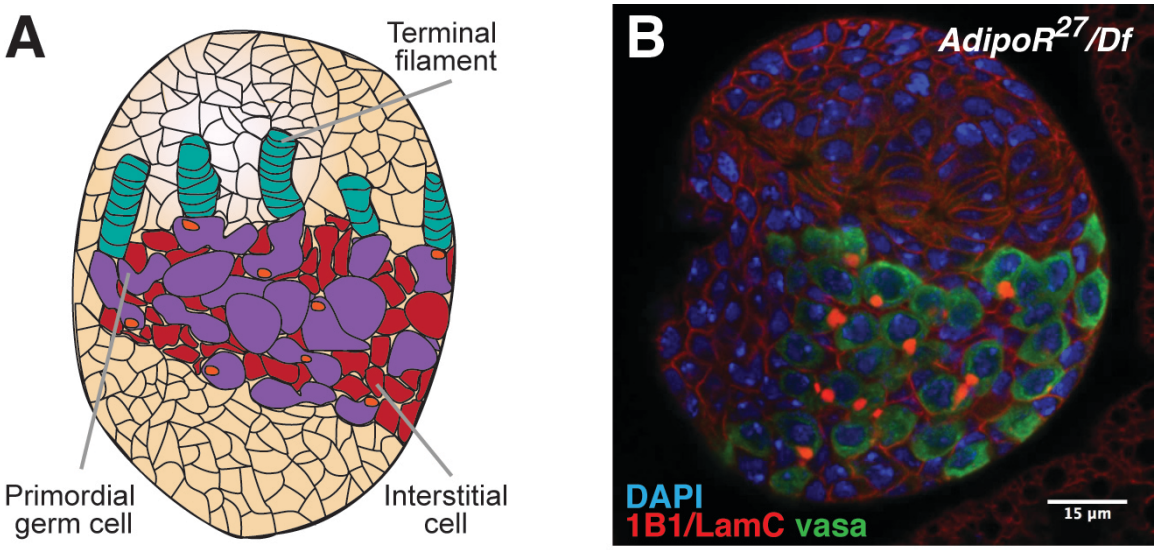


Figure 6.1. AdipoR does not appear to be required for germline progenitor proliferation. (A) The late third instar larval ovary contains mostly undifferentiated primordial germ cells (purple) in association with somatic interstitial cells (red) and newly organized terminal filament cells (teal). The fusome (orange) branches as cells divide and differentiate. **(B)** A representative *AdipoR*^{27} larval ovary, which appears grossly normal. Vasa (green) labels germ cells; 1B1 (red) labels fusome and cell membranes; DAPI (blue) labels nuclei.

(Gilboa 2015). ILPs directly control germline progenitor proliferation, ultimately establishing the size of the GSC pool in adult flies (Gancz and Gilboa 2013). In adult ovaries, ILPs act directly on post-mitotic cap cells to mediate GSC-niche adhesion and niche maintenance (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011, Hsu et al. 2008, Bonfini et al. 2015).

AdipoR^{27} flies do not eclose from their pupal cases, indicating a requirement for adiponectin signaling during development. To test whether *AdipoR* sensitizes the larval germline progenitors to ILPs, I evaluated *AdipoR*^{27} ovaries for gross morphological defects (Figure 6.1B). I did not observe an obvious reduction in the size of the germline progenitor population (n=5), suggesting that *AdipoR* is not required for ILPs to control proliferation of the germline progenitor pool. This superficial analysis does not rule out the possibility of a subtle role for AdipoR signaling in controlling ovarian development, although other obvious roles, including one in terminal filament organization, can be excluded.

Is *AdipoR* required in adult ovarian niche cells to promote GSC maintenance? I used the UAS/GAL4 system (Brand and Perrimon 1993) to specifically express *UAS-RNAi* transgene targeting AdipoR in ovarian somatic cells. I restricted knockdown to adult flies with the use of the temperature sensitive *Gal80* allele, which inhibits Gal4 activity when flies are cultured at its permissive temperature (Figure 6.2; see Methods). Preliminary data suggest that AdipoR is dispensable in cap and escort cells for GSC maintenance (Figure 6.3), and germaria appeared otherwise normal. I exclude the possibility that inefficient knockdown of AdipoR in these cells is responsible for the lack of a phenotype since this *UAS-AdipoR RNAi* line induced phenotypes with other drivers (Figures 6.4 and 6.6). However, since the driver used in these experiments, *c587-Gal4*, expresses only weakly in cap cells (Figure

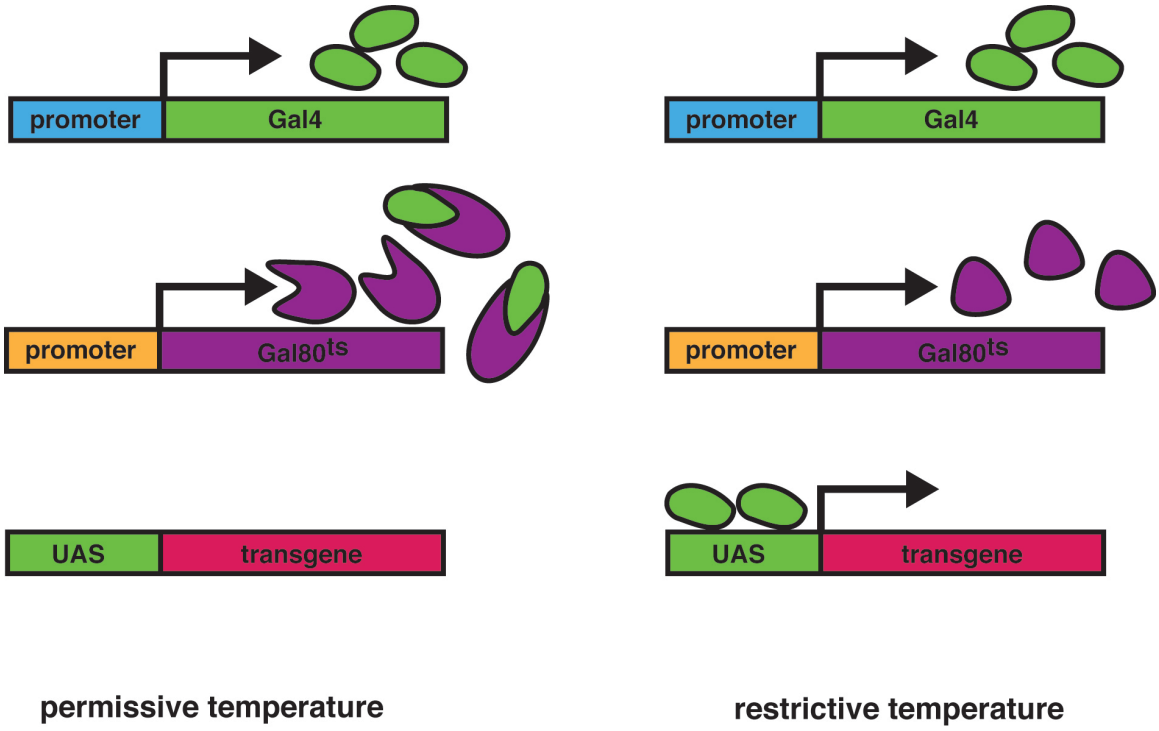


Figure 6.2. The GAL4/UAS system provides spatial and temporal regulation of transgene expression. At the permissive temperature (left), $Gal80^{ts}$ binds to Gal4, inhibiting Gal4-mediated transcription of a transgene with an upstream activating sequence (UAS) promoter. When shifted to the restrictive temperature (right), $Gal80^{ts}$ undergoes a conformational change. Gal4 binds to the UAS, and the transgene is expressed. Adapted from Brand and Perrimon (1993).

6.3A), the major constituent of the GSC niche, multiple driver lines should be used to test the role of AdipoR in ovarian somatic cells before dismissing the possibility of a role in the niche. Taken together, these data suggest that AdipoR is not required for other insulin-dependent processes in the developing and adult ovary, although a definitive conclusion would require additional experiments.

Preliminary studies suggest an ovary non-autonomous role for AdipoR in controlling germline cyst division and progression through vitellogenesis

The ovary is not the exclusive insulin-sensitive tissue in the fly. During development, the fat body itself responds to insulin signals to regulate organismal growth (Britton et al. 2002, Hyun et al. 2009), insulin signaling regulates lipid storage in the adult fat body (DiAngelo and Birnbaum 2009), and unpublished work in our lab indicates that adipocyte insulin signaling is required for progression through vitellogenesis (Alissa Armstrong, unpublished observations). The presence of AdipoR transcripts in adult abdominal carcasses suggests that it is present in the fat body (Figure 3.1). Does AdipoR function in the fat body affect oogenesis?

Again using the UAS/GAL4 system (Figure 6.2), I investigated the role of AdipoR in the adult fat body. Previously, Leesa LaFever Sampson observed that knockdown of AdipoR with *FB-Gal4* (Grönke et al. 2003) leads to one additional round of cell division in some germline cysts, generating oocytes with five ring canals and follicles with 32-cell cysts. I was able to reproduce this phenotype (Figure 6.4). Interestingly, I did not observe 32-cell cysts when knocking down AdipoR with a different fat body driver, *3.1Lsp2-Gal4* (Lazareva et al. 2007) (Figure 6.4). The difference in phenotype could be due to driver strength, or, more

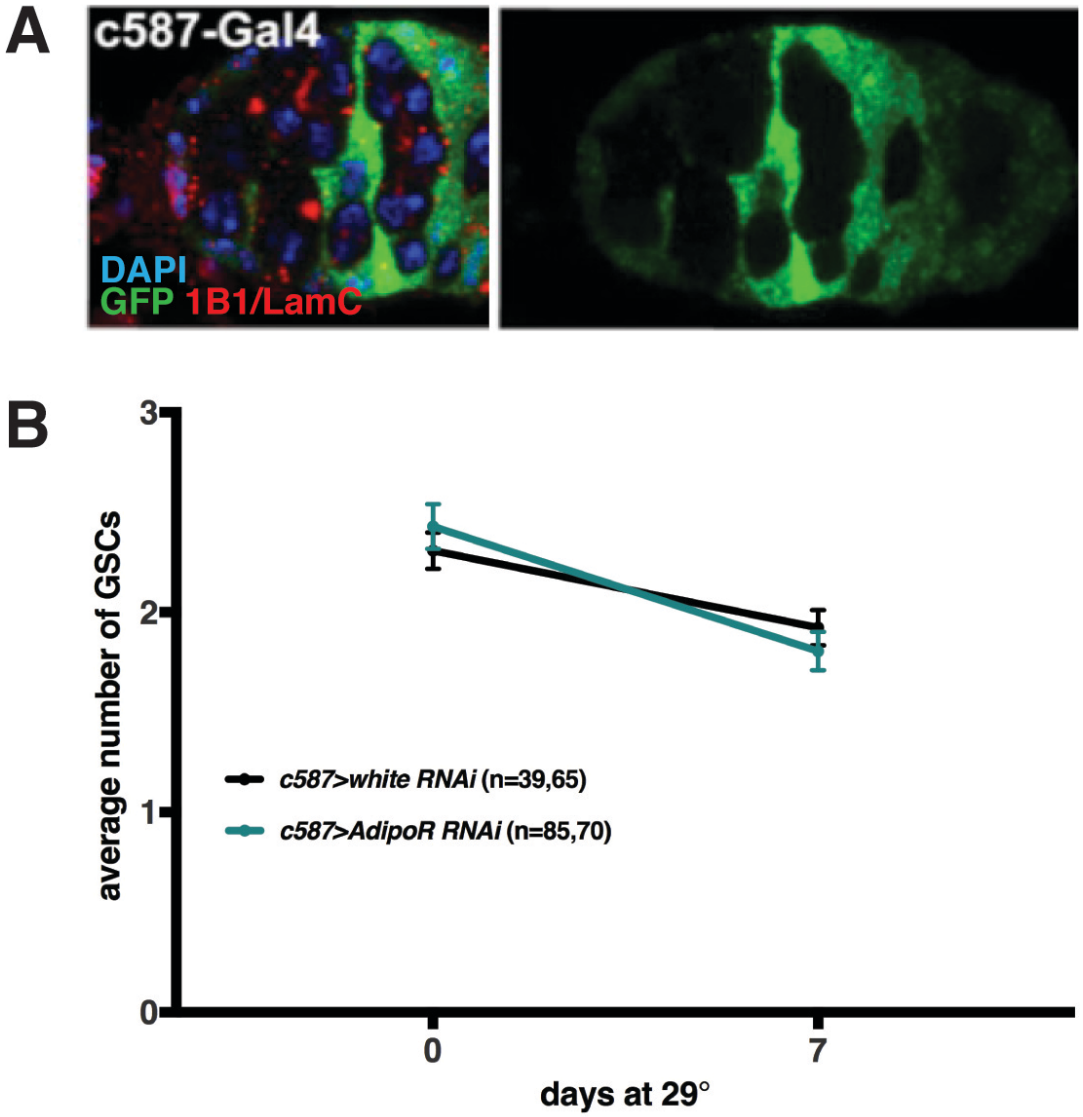


Figure 6.3. AdipoR knockdown in somatic cells in the germarium does not affect GSC maintenance. (A) Expression of *UAS-GFP* (green) induced by *c587-Gal4* in adult females demonstrates low driver expression in cap and early escort cells and strong expression in later escort and follicle cells. DAPI (blue) labels nuclei; GFP (green) labels cells expressing c587-Gal4; 1B1 (red) labels fusome and cell membranes; LamC (red) labels cap cells. Image at right shows only GFP in the same germarium. Images courtesy of Alissa Armstrong. **(B)** Average number of GSCs per germarium at zero and 10 days of *c587; Gal80^{ts}*-mediated induction of RNAi transgene against AdipoR or white control. There is no statistically significant difference by two-way ANOVA with interaction. Samples sizes are indicated with each genotype. Error bars indicate mean $\pm$ s.e.m.

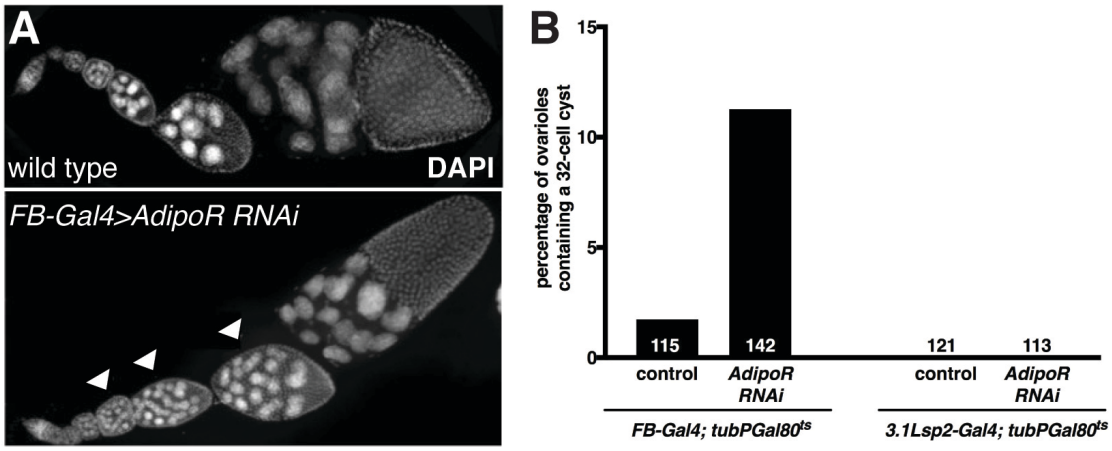


Figure 6.4. AdipoR knockdown with *FB-Gal4*, but not *3.1Lsp2-Gal4*, induces 32-cell cysts in ovaries. (A) In wild type ovarioles (above), germline cysts contain 16 cells. *FB-Gal4*-mediated knockdown of AdipoR (below) generates ovarioles with 32-cell cysts (arrowheads; a dramatic example is shown). DAPI (grey) stains nuclei. *FB-Gal4* image courtesy of Leesa Sampson. **(B)** Quantification of ovarioles containing 32-cell cysts in sibling controls and *AdipoR RNAi* females. Sample sizes are indicated on bars and reflect the number of ovarioles observed in a single experiment.

likely, disparate driver expression patterns. Careful analysis of a panel of putative fat body drivers by Alissa Armstrong, a postdoctoral fellow in the lab, demonstrated that while *3.1Lsp2-Gal4* expression is restricted to adult adipocytes, *FB-Gal4* expresses in several adult tissues, including oenocytes and some cells in the gut (Figure 6.5). Based on this result, we speculate that signals from oenocytes or specific gut cells could control the cell cycle of germline cysts. Future studies with drivers that express in these tissues could pinpoint the origin of that signal. Preliminary data further suggest that adipocyte AdipoR also promotes progression through vitellogenesis, as *3.1Lsp2-Gal4*-mediated knockdown of AdipoR leads to a dramatic increase in the number of ovarioles with degenerating vitellogenic egg chambers (Figure 6.6). One possible explanation for this effect is that unlike in the germline or somatic cells of the ovary, AdipoR promotes insulin sensitivity in adult adipocytes; insulin signaling in several species promotes vitellogenin transcription in the fat body (Roy et al. 2007, Parthasarathy and Palli 2011, Badisco et al. 2011, Gulia-Nuss et al. 2011, Abrisqueta et al. 2014), and adipocyte insulin signaling regulates vitellogenesis (Alissa Armstrong, unpublished observations). That a block to vitellogenesis is exclusive to *3.1Lsp2-Gal4*-mediated AdipoR knockdown and is not observed in *FB-Gal4 AdipoR-RNAi* flies (data not shown) suggests a difference in the expression of these drivers in adult adipocytes; indeed, our visual expression analysis indicates that *3.1Lsp2-Gal4* is more strongly expressed in adult female adipocytes than *FB-Gal4* (Figure 6.5). These preliminary results point to several potential ovary non-autonomous roles for AdipoR, all of which require further investigation.

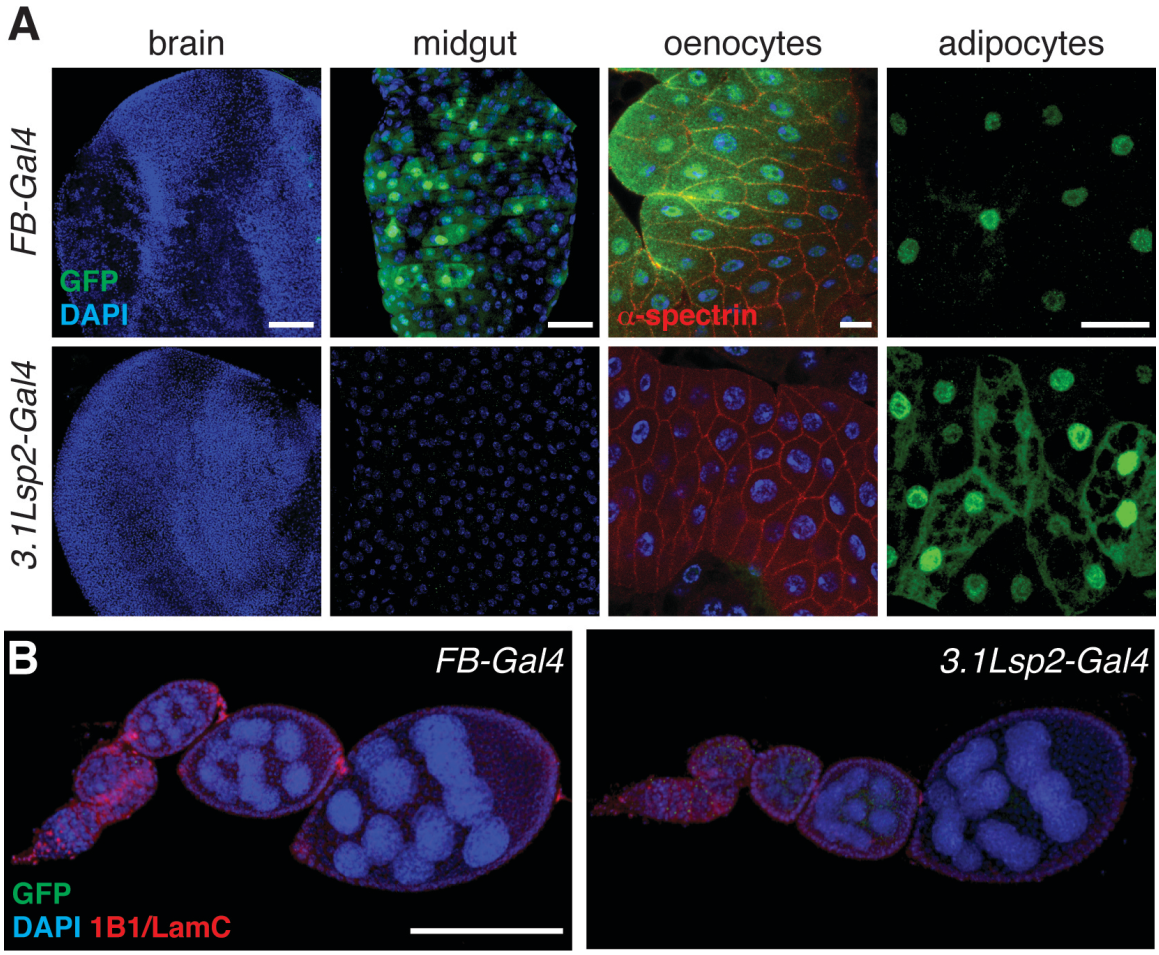


Figure 6.5. In adult females, *3.1Lsp2-Gal4*, but not *FB-Gal4*, is expressed exclusively in adipocytes. **(A)** Expression of *UAS-GFP* (green) induced by *FB-Gal4* or *3.1Lsp2-Gal4* in the brain, midgut, oenocytes, and adipocytes of well-fed adult females. **(B)** There is no *UAS-GFP* expression in the ovaries of *FB-Gal4* or *3.1Lsp2-Gal4* flies. DAPI (blue) labels nuclei in brains, guts, oenocytes, and ovaries; α-spectrin (red) labels cell membranes in oenocytes; 1B1 (red) labels cell membranes in ovaries; LamC (red) labels cap cells in ovaries. Scale bars: 50 μm (brains and guts), 10 μm (oenocytes), 20 μm (adipocytes), or 100 μm (ovaries). Figure is adapted from Armstrong et al. (2014), and portions of it appear in Figures 5.2 and 5.3.

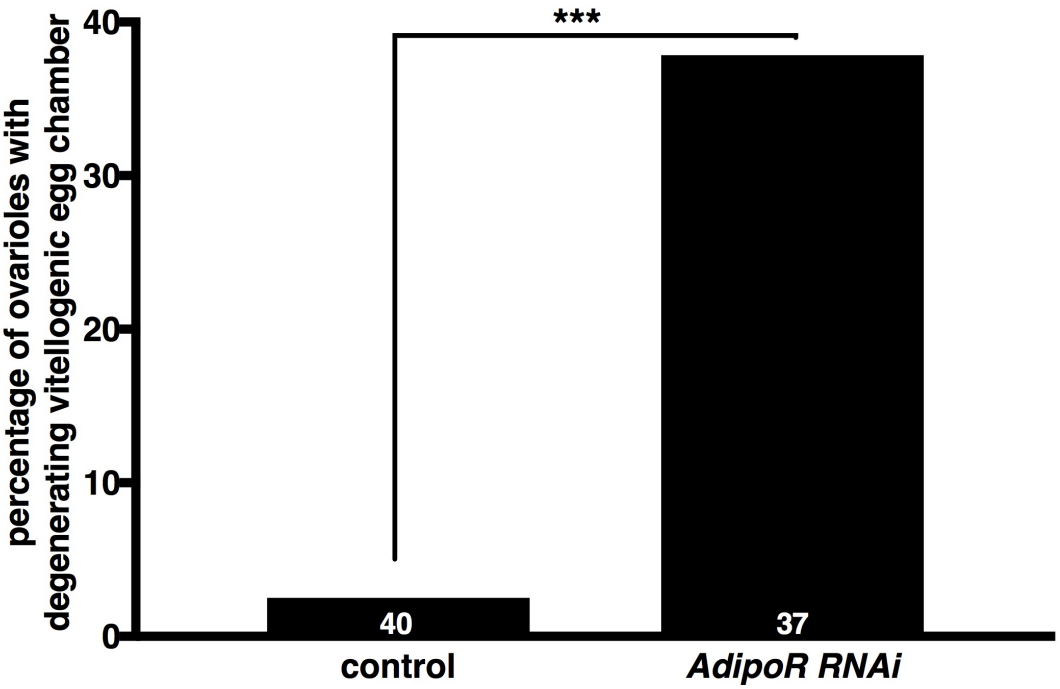


Figure 6.6. Adipocyte AdipoR appears to support progression through vitellogenesis. Percentage of ovarioles containing degenerating vitellogenic egg chambers at 7 days of adipocyte-specific knockdown of AdipoR. ***P<0.001 by Chi-square. Sample size from a single experiment is show in each bar.

Attempts to lineage label the adult fat body

Although RNAi is a powerful technique, its use comes with substantial caveats. Concerns about knockdown efficacy and off-target effects can be addressed through the use of multiple RNAi lines or by reproducing results with alternative genetic techniques. As illustrated in Chapters II-IV, generating genetic mosaic animals allows comparison of mutant and wild type cells within the same tissue and the investigation of cell-autonomous roles for genes, both while circumventing developmental lethality. Furthermore, if the right developmental induction point is selected, very large clones can be made. While mosaic analysis is commonly used in larval fat body studies, its use in adult adipocytes has not been described. FLP/*FRT*-mediated mosaic analysis depends on cell proliferation to generate visually identifiable clones (see Chapter II for details). The larval fat body is extensively remodeled during metamorphosis (Nelliot et al. 2006), then histolyzed and completely replaced by adult tissue three days after eclosion (Postlethwait and Jones 1978). The timeline of proliferative expansion of adult adipocyte precursors, however, is unknown. To describe the proliferative window these precursors and to develop a protocol to generate adult mosaic adipocytes, I used a heat-shock inducible *LacZ* lineage tracing system [Figure 6.7; (Harrison and Perrimon 1993)] to positively mark mitotically active cells. Previous studies have proposed that adult fat cells come from a small, mitotically active precursor population that expands during the first few days after eclosion, while the larval fat body is still present (Hoshizaki et al. 1995, Aguila et al. 2007). When heat shocking flies during the third larval instar, when imaginal discs are mitotically active, a previous graduate student in the lab, Leesa LaFever Sampson, had modest success in positively marking small numbers of adult adipocytes (1-2 cells). Since this does not account for the entire adult adipocyte

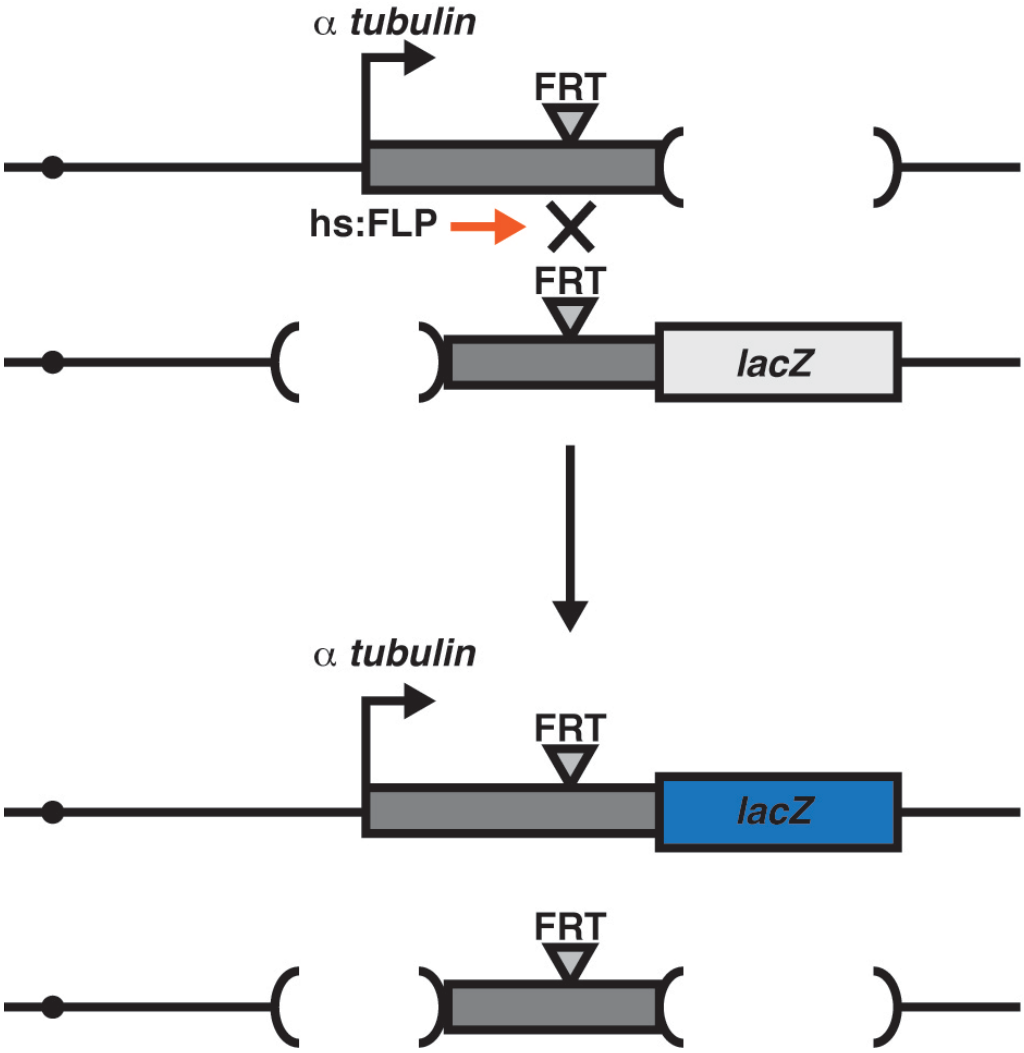


Figure 6.7. A LacZ lineage tracing system. An *α-tubulin* promoter and *lacZ* transgene in *trans* do not express β-galactosidase (above). Following heat shock, FLP/*FRT*-mediated mitotic combination occurs in some cells, and a functional *tubulin-lacZ* is reconstituted (below, in blue). Positive marking by constitutive expression of β-galactosidase allows lineage tracing. Modified from Drummond-Barbosa and Spradling (2001).

lineage, we proposed to heat shock cells at various points during development to induce β-gal expression in proliferative cells (see Methods). Despite reliably detecting positive marks in the ovary, indicating that the lineage tracing was working, I did not reproducibly detect β-gal -positive cells in the adult fat body (Figure 6.8). This was in contrast to preliminary studies, where I observed β-gal-positive cells in adult flies that had been heat shocked as embryos. Therefore, a mosaic analysis protocol was not developed for the adult fat body, and *AdipoR*^{27} adult adipocytes were not generated to further investigate the role for AdipoR in that tissue.

A candidate approach for identifying the *Drosophila* adiponectin-like ligand

AdipoR intrinsically controls GSC maintenance (Chapter III), and identifying its ligand could shed light on mechanisms of adipokine signaling in flies. While there is no *Drosophila* homolog for mammalian adiponectin on the primary sequence level, the fly AdipoR ligand could structurally resemble mammalian adiponectin. Indeed, the tobacco plant encodes a homolog of adiponectin, osmotin, which has no sequence similarity to the mammalian protein but can bind the yeast adiponectin receptor and induce apoptosis (Narasimhan et al. 2001). Structural analysis demonstrated that adiponectin and osmotin share a lectin-like domain (Narasimhan et al. 2005), making *Drosophila* proteins with this domain interesting candidate ligands for AdipoR.

A family of putative juvenile hormone binding proteins, all encoding uncharacterized, short peptides of about 300 amino acids, is predicted to have a lectin-like domain. One member, CG8997, interacts with AdipoR in a yeast two-hybrid assay (www.ebi.ack.uk/inact/). In conjunction with two rotation students, Lyle MacPherson and

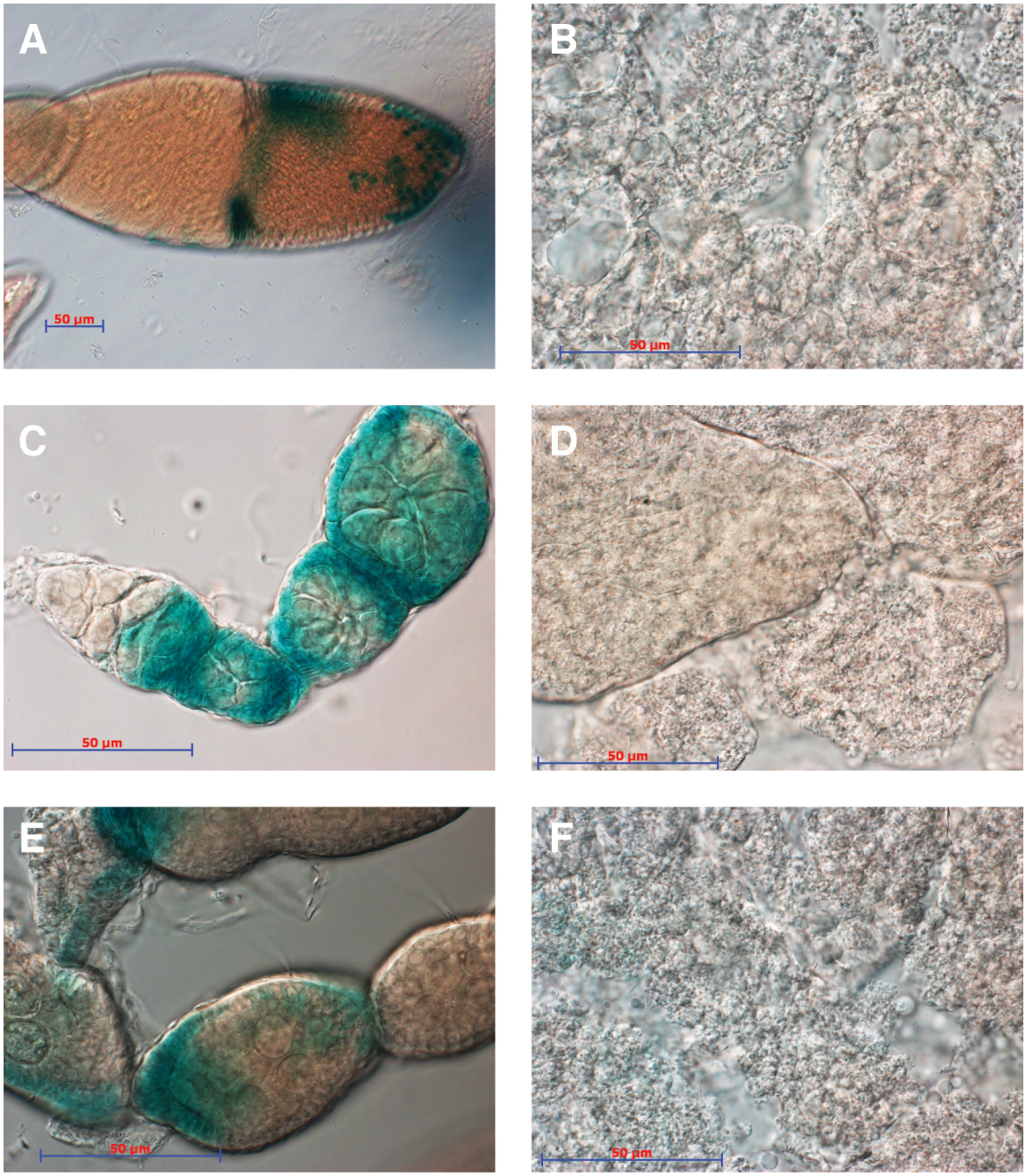


Figure 6.8. Induction of the LacZ lineage tracing system from 1-3 days after egg laying does not consistently label the adult fat body. (A and B) Representative images of an ovary (A) and adipocytes (B) from flies exposed to a developmental heat shock at 1 day after egg laying. **(C and D)** Representative images of an ovary (C) and adipocytes (D) from flies heat shocked at 2 days of development. **(E and F)** Representative images of an ovary (E) and adipocytes (F) from flies heat shocked at 3 days of development Scale bars, 50 µm.

Edward Culbertson, I characterized the expression pattern of these five candidate ligands in wild type *Drosophila* adult females by RT-PCR (Figure 6.9). Transcripts of all but one candidate ligand were detected in whole fly extracts, and all transcripts were notably enriched in the gut. None of the ligands are robustly expressed in the ovary or head, but we detected transcript in the thorax, representing mostly muscle tissue, and abdominal carcass, representing mostly the fat body.

Since *AdipoR* is required for GSC maintenance (Chapter III), we predicted that mutation or knockdown of a *bona fide* adiponectin-like ligand would lead to GSC loss in adult flies. Using a ubiquitous somatic driver, *act5C-Gal4*, we knocked down each candidate ligand and evaluated GSC number over time (Figure 6.10, 6.11). While GSC number decreases over time when candidate ligands are globally knocked down, GSC loss is statistically comparable to that in controls, indicating that the manipulation has no effect on GSC maintenance. This negative result, however, does not eliminate the possibility that these candidates genetically interact with *AdipoR*. The candidate ligands may be functionally redundant, making it necessary to knock down several simultaneously to observe a phenotype. Further experiments with several drivers and evaluation of knockdown efficacy would also help determine whether these proteins are involved in *Drosophila* adiponectin signaling. Genetic interaction and biochemical binding assays would be necessary to definitively determine whether any of these is a *bona fide* AdipoR ligand. Therefore, the identity of the adiponectin-like ligand in *Drosophila* remains unclear.

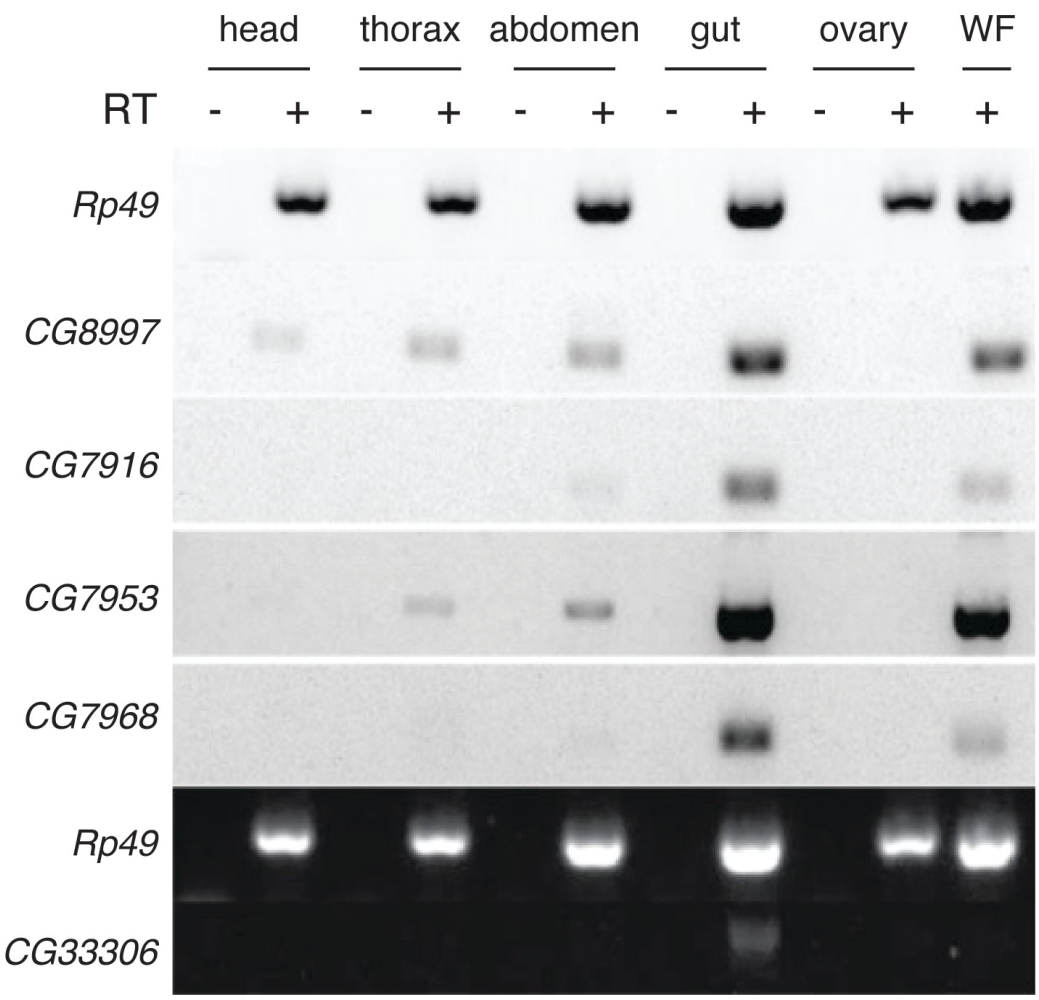


Figure 6.9. Candidate adiponectin-like ligand transcription is enriched in the adult gut. RT-PCR analysis of candidate ligand gene expression in adult head, thorax, abdomen, gut, and whole *yw* female flies. *Rp49* is a control. –RT, negative control with no reverse transcriptase in RT reaction.

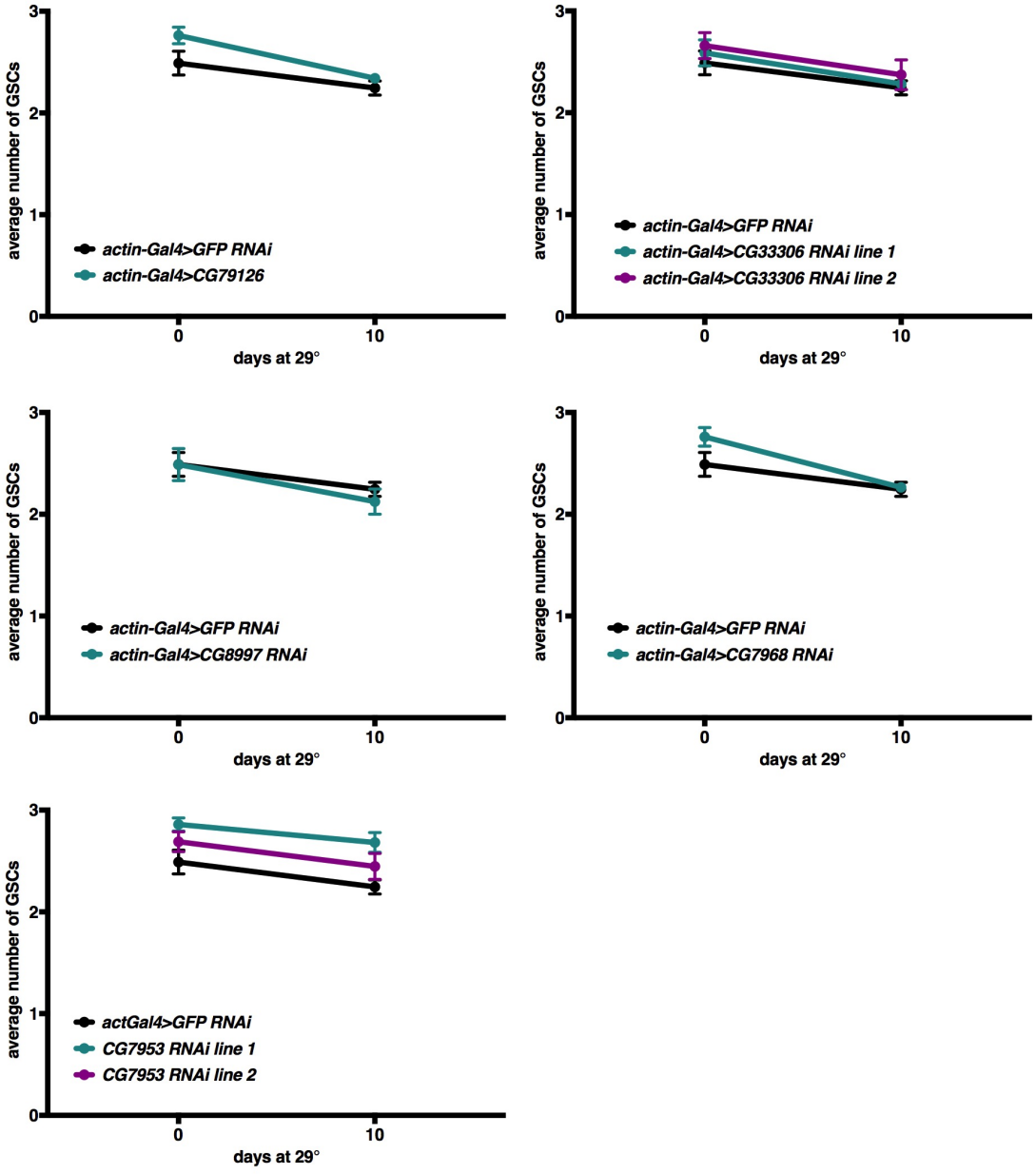


Figure 6.10. Candidate adiponectin-like ligands do not appear to control GSC maintenance. Average number of GSCs per germarium at zero and 10 days of *act5C-Gal4*-mediated induction of *UAS-RNAi* transgenes targeted candidate adiponectin-like ligands or *GFP RNAi* control. All experiments were performed simultaneously and graphed separately for clearer visualization. See Figure 6.11 for samples sizes and distribution. Error bars represent mean ± s.e.m. over three independent trials. No significant difference in GSC loss by two-way ANOVA with interaction.

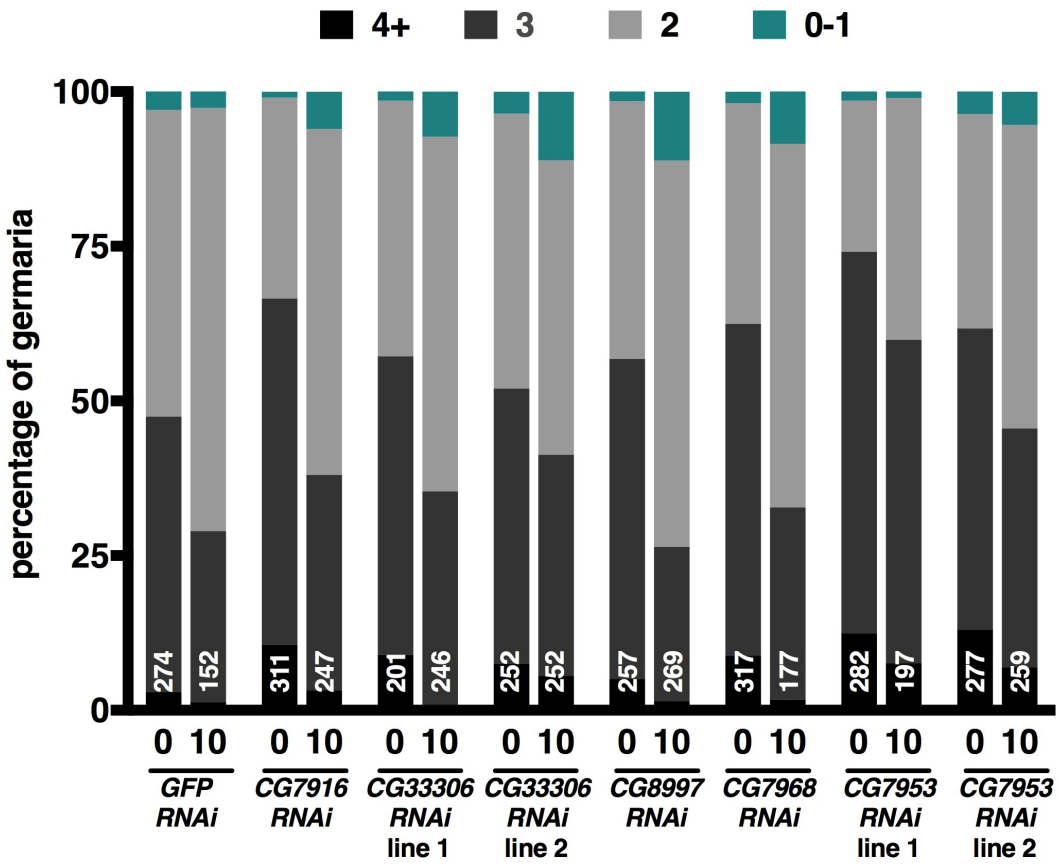


Figure 6.11. Candidate adiponectin-like ligands do not appear to control GSC maintenance. Frequencies of germaria containing zero-or-one, two, three, or four or more GSCs at different times after switch to 29°C for *act5C-Gal4* mediated induction of candidate ligand *UAS-RNAi* transgenes or *GFP RNAi* control. The same data used to calculate GSC number averages in Figure 6.10 are shown. The number of germaria analyzed is indicated on each bar and represents combined data from three independent experiments.

Lipid storage mutants appear to have normally proliferating GSCs

In mammals, the level of fat storage influences the circulating levels of adipokines. For example, obese individuals have lower circulating adiponectin and abnormal leptin levels(Ryan et al. 2003). Several genes controlling lipid storage have been identified in *Drosophila*, including *adipose* (*adp*), the homolog of human WD and tetratricopeptide repeats 1 (WDTC1) (Suh et al. 2007), and *lipid storage droplet 2* (*Lsd2*), the homolog of human Perilipin (Grönke et al. 2003, Gronke et al. 2005). Work by Leesa LaFever Sampson, a previous graduate student in the lab, suggested that lipid storage global mutants had aberrant oogenesis. Leesa observed that both $Lsd2^{51}$ mutants, which have reduced levels of lipids due to increase lipase activity, and adp^{60} mutants, which have increased fat stores, lay fewer eggs per day than control flies. Furthermore, she observed changing the branching of the fusome in these mutants. Since the fusome branches between cells in germline cysts, it becomes progressively more reticulated as the cysts divide; the presence of more branched fusomes could indicate a change in differentiation or proliferation of GSCs or their progeny. To directly measure GSC proliferation, I performed an EdU incorporation assay on the ovaries of global lipid storage mutants. Results from a single experiment show no dramatic difference in time spent in S phase between sibling controls and lipid storage mutants (Figure 6.12). While it is possible that lipid storage mutants have normal GSC proliferation, these preliminary data show a subtle downward trend compared to siblings; repeat experiments are necessary to confirm this result. Since I used relatively young flies (four to five days old) in this assay and lipid composition in *Drosophila* changes with aging (Nasiri Moghadam et al. 2015), it would be interesting to test whether mutants maintain wild type levels of GSC proliferation over time.

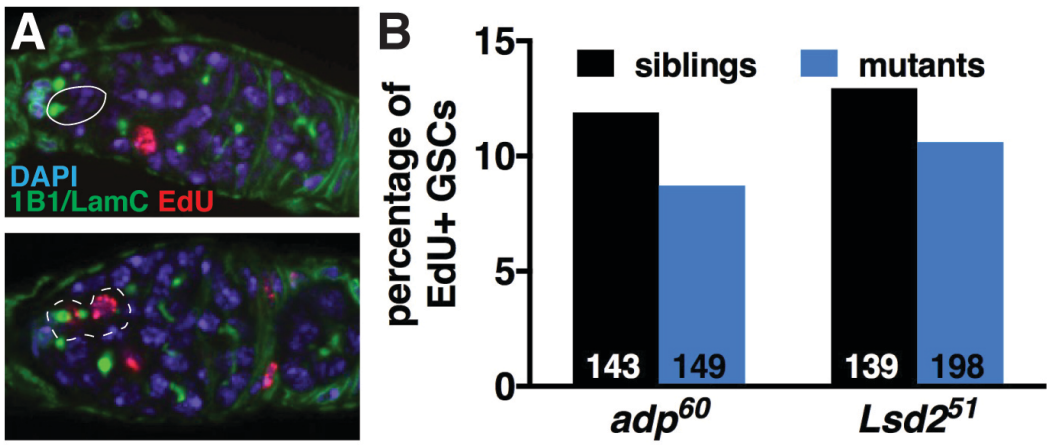


Figure 6.12. Lipid storage mutants do not have altered GSC proliferation. (A) *Lsd2*^{51} mutant germaria containing GSCs without (above, solid line) and with (below, dashed line) EdU incorporation (red). DAPI (blue) labels nuclei, 1B1 (green) labels fusome and cell membranes, Lamin C (LamC, green) labels cap cell nuclear envelopes. Images were acquired at the same magnification. **(B)** Percentage of GSCs that incorporated EdU in *adp*^{60} *and Lsd2*^{51} global mutant germaria and sibling controls. Sample size from a single experiment is indicated in each bar.

Discussion

Adipocyte biology and its effects on organismal physiology can be complex, and the endocrine role of *Drosophila* adipocytes is an active area of investigation. This chapter is a collection of unpublished efforts toward understanding the role of the fat body in regulating *Drosophila* oogenesis. While my data do not support a model where adiponectin signaling controls insulin-dependent processes in the development and activity of the ovary, they do suggest a tissue non-autonomous role for *AdipoR* in controlling oogenesis. Other studies were not extensive enough to conclusively identify the *Drosophila* adiponectin-like ligand or to establish a link between lipid storage and GSC proliferation, which previous work in our lab had suggested.

AdipoR may have insulin-dependent and independent roles

From our previous work, we know that adiponectin-like signaling, which sensitizes peripheral tissues to insulin in mammals (Yamauchi and Kadowaki 2013), has an insulin-independent role in *Drosophila* ovarian GSC maintenance (Chapter III). My preliminary data suggest that AdipoR does not control germline progenitor proliferation or niche-mediated GSC maintenance in adult females, both of which are insulin-dependent processes. However, the apparent requirement of adipocyte AdipoR for progression through vitellogenesis could reflect a connection to insulin signaling. Knockdown of the insulin receptor in the German cockroach *Blattella germanica*, for example, reduces vitellogen synthesis in the fat body, potentially compromising yolk uptake and progression through vitellogenesis (Abrisqueta et al. 2014). Indeed, this is a common effect in insects, observed in red flour beatles (Parthasarathy and Palli 2011), desert locusts (Badisco et al. 2011), and the yellow fever mosquito, *Aedes aegypti* (Gulia-Nuss 2011 and Roy 2007). Unpublished data

from our lab indicates that insulin signaling in adipocytes controls progression through vitellogenesis (Alissa Armstrong, unpublished data). Further preliminary data indicate that AdipoR is required tissue non-autonomously to control the number of germline cyst divisions in the ovary. Extra rounds of cell division have been observed in flies with aberrant germline cell cycles, specifically those with untimely degradation or overexpression of cyclins E or A (Ohlmeyer and Schupbach 2003, Lilly and Spradling 1996, Lilly et al. 2000). Mutations in *fused*, a receptor tyrosine kinase that regulates BMP signaling (Xia et al. 2010), also lead to an extra round of cell division in female germline cysts (Narbonne-Reveau et al. 2006). Whether AdipoR non-autonomously controls the germline activity in the germarium through these established mechanisms would be an interesting future direction.

Identifying adiponectin-like ligands in *Drosophila*

In mammals, adiponectin is the most abundant transcript in adipocytes (Maeda et al. 1996); it is not, however, exclusively produced in adipose tissue (Delaigle et al. 2006, Krause et al. 2008), and it is possible that the adiponectin-like ligand in *Drosophila* is not produced by adipocytes at all. While my attempt to identify adiponectin-like ligands in *Drosophila* was incomplete, there are several additional approaches that could be taken to identify constituents of the AdipoR signaling cascade. For example, some groups have successfully identified G-protein coupled receptor ligands based on mRNA screens, taking advantage of the reciprocal transcriptional regulation of ligands and receptors *in vivo* to identify candidate ligands in receptor knockdown backgrounds (Alfa et al. 2015). More generally, a dominant genetic interactor screen could be performed, taking advantage of the homozygous lethality of $AdipoR^{27}$ and the large collection of molecularly defined deficiencies available in *Drosophila*. Finally, the many signaling pathways implicated in adiponectin signaling in *in*

vitro systems, including calcium and ceramidase signaling (Iwabu et al. 2010, Holland et al. 2011), could be investigated in a candidate approach to identify downstream components of AdipoR signaling in *Drosophila*.

Hurdles to lineage tracing the adult fat body

Outside of possible technical problems, mosaicism could be difficult to induce for biological reasons. For example, rather than expanding their population through a single burst of proliferation at one point in development, adult adipocytes precursors could undergo several rounds of limited proliferation over the course of development. The observation of adult fat body expansion at different developmental time points supports this model. The larval fat body is highly regionalized (Haunerland 1995), and the adult fat body may also have distinct regions that serve different purposes; perhaps different regions of the fat body arise from independent precursor populations. Future studies using the *LacZ* lineage tracing system for adult adipocyte lineage tracing should test this model and address potential technical difficulties, including visibility of staining in adult adipocytes and effectiveness of heat shocks. Alternately, flipase expression could be regulated by a driver instead of by heat shock. For this method to be effective the driver would need to be transcribed in the early stages of adult adipocyte precursor proliferation; to my knowledge, no such tool has yet been described.

CHAPTER VII

DISCUSSION AND FUTURE DIRECTIONS

In adults, stem cells act to maintain tissue function in response to injury, and the systemic environment (Ables et al. 2012). The focus of this dissertation is how *Drosophila* ovarian stem cell lineages, which have well-characterized responses to diet (Chapter I), sense and respond to their physiological environment. How does diet affect the whole-body physiology of the fly, and how is that information relayed to the ovary? Multiple organs are involved in attuning the ovary to the nutrient status of the organism. For example, insulin-like peptides (ILPs) secreted from the brain act directly on the germline to control germline stem cell (GSC) proliferation and progeny growth and survival (LaFever and Drummond-Barbosa 2005), and amino acid sensing in adipocytes supports GSC maintenance (Chapter V). Diet may also be sensed by signaling relays, as when well-fed flies secrete leptin-like ligand unpaired-2 (upd-2) from their fat bodies, stimulating ILP secretion from the brain (Rajan and Perrimon 2012). Furthermore, successful oogenesis may feed back onto itself, as ecdysone, produced by late-stage ovarian follicles in *Drosophila*, also acts on GSCs to promote their maintenance (Ables and Drummond-Barbosa 2010). While the contributions of each constituent may be relatively small in a dynamic physiological environment, the overall effect of this signaling milieu is to precisely regulate stem cell lineages in concert with small variations in nutrient state.

This work has substantially contributed to the understanding of this physiological regulation of ovarian stem cell lineages. At its onset, the role of the larval fat body in regulating organismal growth and the immune response was well established (Colombani et

al. 2003, Kounatidis and Ligoxygakis 2012), and a series of studies had solidified the importance of the fat body in mediating the ovarian response to blood feeding in anautogenous mosquitoes (Attardo et al. 2005, Attardo et al. 2006, Hansen et al. 2004, Hansen et al. 2005, Martin et al. 2001, Pierceall et al. 1999, Raikhel 1986, Raikhel and Dhadialla 1992, Roy et al. 2007). While adult *Drosophila* fat body function had been implicated in male courtship behavior (Lazareva et al. 2007) and female yolk protein production (Bownes and Hames 1977), rigorous genetic studies characterizing the contribution of the adult fat body to organismal physiology were limited, and how those changes might affect oogenesis was unknown. I used the genetic tools available in *Drosophila melanogaster* to explore how stem cell lineages in the adult ovary coordinate their activity with the physiological environment as sensed by the ovary itself (Chapters III and IV) and as communicated to the ovary by the fat body (Chapters V and VI). Thus, this work represents a major step toward understanding the dietary and physiological control of insect oogenesis and adult stem cell populations.

In this final chapter, I extend the discussion started in previous chapters and describe potential future directions for this work.

Approaches to uncovering the *Drosophila* adiponectin receptor signaling axis

In Chapter III, I demonstrate that the *Drosophila* homolog of the adiponectin receptor, AdipoR, has an intrinsic, insulin-independent role in regulating GSC maintenance. AdipoR influences GSC adhesion to cap cells by modulating E-cadherin levels and is required for a robust GSC response to bone morphogenic protein (BMP) ligands, which suppress GSC differentiation (Xie and Spradling 1998) (Figure 3.8). These small, but

statistically significant, effects likely do not explain the extent of GSC loss in *AdipoR* mutant mosaic germaria, and uncovering the players in adiponectin signaling is an important future direction for this research.

What are the other constituents of the AdipoR signaling cascade in *Drosophila*? We reason that, like *AdipoR*, its signaling pathway components will be required for GSC maintenance. The abundance of studies describing mammalian adiponectin signaling in cell and *ex vivo* cultures provides a trove of candidate interactors in *Drosophila*. In Chapter VI, I outline an approach to evaluate those pathways for a role in *Drosophila* AdipoR signaling as well as an unbiased dominant genetic interaction screen. Furthermore, I use an RNAi approach to test a family of candidate ligands that share a lectin-like domain with mammalian adiponectin, but see no statistically significant difference between their stem cell maintenance over time and that of control samples. This does not necessarily eliminate these candidates from contention; only one driver was tested, and I did not evaluate knockdown efficacy. Furthermore, ligands could be redundant, and knocking down a single candidate might not be sufficient to significantly disrupt signaling. One way to address this possibility is to remove the gene cluster encoding these five closely-related genes using the CRISPR/Cas9 system, for which many tools are available in flies (Xu et al. 2015).

AdipoR mutant GSCs are lost from the niche nearly five times as frequently as control GSCs in well-fed flies, but only twice as frequently on a poor diet, suggesting that AdipoR signaling receives dietary input (Figure 3.5). qPCR experiments demonstrate that ovarian AdipoR transcription is not regulated by diet (Figure 3.6), leaving open the possibility of, among other things, dietary regulation of ligand transcription or secretion. With the goal of determining differences in the adult fat body proteome in flies fed rich and poor diets, we

performed mass spectrometry experiments. With Leesa LaFever Sampson, I manually dissected the fat bodies of four biological replicates of approximately one hundred wild-type flies fed either a rich diet or shifted to a poor diet for 12 hours prior to dissection. We extracted protein from these fat bodies and submitted our samples to the Mass Spectrometry Core at the Johns Hopkins School of Medicine for comparison using isobaric tags for relative and absolute quantitation (iTRAQ), under the supervision of Dr. Bob Cole. The analysis detected 2,525 fat body proteins, 226 of which were more abundant in the fat body on a poor diet and 224 of which were less abundant (Matsuoka et al., in preparation). In addition to identifying candidates for regulating the ovarian response to diet, the iTRAQ data set may contain the adiponectin-like ligand (although the limited sensitivity of iTRAQ may mean our data set is missing many small secreted proteins). Shinya Matsuoka, a current postdoctoral fellow in the lab, is preparing a manuscript detailing his analysis of the iTRAQ dataset and studies of candidates identified by this experiment in the ovarian response to diet.

That the *Drosophila* adiponectin-like ligand is secreted from the fat body, however, should not be a foregone conclusion. Indeed, although adiponectin is the most abundant transcript in mammalian adipocytes (Maeda et al. 1996), it is not exclusively produced in fat tissue (Delaigle et al. 2006, Krause et al. 2008). Furthermore, *Caenorhabditis elegans*, which encodes three AdipoR homologs, *paqr-1,-2,* and *-3*, do not have a dedicated lipid storage organ, instead storing lipids primarily in their intestine and epidermis (Mullaney and Ashrafi 2009). Any approach to finding the adiponectin-like ligand should, therefore, consider multiple organs as its potential origin.

AdipoR is required during development for survival (Table 3.1), and *AdipoR* mutants fail to eclose from their pupal cases. AdipoR is dispensable, however, for ovarian

development through the later third larval instar (Figure 6.1). What is the role for AdipoR in *Drosophila* development? The death of *AdipoR*^{27} mutants at the pharate stage is not wholly unusual, but is reminiscent of mutations in the gene encoding *Drosophila* Hepatic nuclear receptor 4 (dHNF4), a nuclear hormone receptor that controls lipid metabolism (Palanker et al. 2009), raising the interesting possibility that adiponectin also modulates this process in *Drosophila*. Adiponectin regulates circulating lipid levels in humans (Baratta et al. 2004), and influences lipid metabolism in mouse skeletal muscle and bovine hepatocytes (Staiger et al. 2004, Chen et al. 2013). Future studies should evaluate the lipid content of *AdipoR*^{27} mutants as a preliminary step toward understanding its role in this context.

While we have clearly demonstrated an insulin-independent role for AdipoR in GSCs (Chapter III), AdipoR could still be involved in sensitizing other tissues to insulin. In Chapter VI, I describe preliminary data from studies testing whether AdipoR is required in the somatic niche in the ovary, where ILPs act to control GSC maintenance (Hsu and Drummond-Barbosa 2009, Hsu and Drummond-Barbosa 2011), or in the adult fat body. While preliminary data suggest that AdipoR is dispensable in cap cells (Figure 6.3), fat body knockdown of AdipoR causes two distinct ovarian phenotypes: *FB-Gal4*-mediated knockdown of AdipoR generated follicles with 32-cell cysts (Figure 6.4), and *3.1Lsp2-Gal4*-mediated knockdown lead to a block to vitellogenesis (Figure 6.6). *FB-Gal4* and *3.1Lsp2-Gal4* are both expressed in adult female adipocytes, but *FB-Gal4* has a broader expression pattern comprising oenocytes and a subset of cells in the gut (Figure 6.5). The fact that *FB-Gal4*-mediated AdipoR knockdown does not cause a block in vitellogenesis, while *3.1Lsp2-Gal4*-mediated knockdown does (Figure 6.6) likely reflects the weaker adipocyte expression of the former (Figure 6.5). *FB-Gal4* expression outside of adipocytes is likely responsible

for the cell cycle effect. Whether AdipoR remotely modulates the cell cycle in the early germarium by its action in the gut or in oenocytes will require the use of additional drivers with narrow expression patterns, or expression patterns that overlap selectively with that of *FB-Gal4*. An extra round of germline mitoses could be the consequence of delayed germline abcission, as has been described in cell cycle and BMP regulation mutants (Ohlmeyer and Schupbach 2003, Lilly et al. 2000, Lilly and Spradling 1996, Narbonne-Reveau et al. 2006). If and how AdipoR remotely regulates the cell cycle in the GSC lineage would be an interesting question for future study. Additionally, how AdipoR non-autonomously controls progression through vitellogenesis could shed light on adiponectin signaling in adipocytes. Through the use of an insulin signaling reporter, such as pleckstrin homology domain-GFP (Britton et al. 2002), we could determine if adipocyte insulin signaling were disrupted in *3.1Lsp2-Gal4*-mediated AdipoR knockdown flies. Is AdipoR required in adipocytes for the transcription of vitellogenin, or yolk proteins, as it is in other insect species (Roy et al. 2007, Parthasarathy and Palli 2011, Badisco et al. 2011, Gulia-Nuss et al. 2011, Abrisqueta et al. 2014), or does it act to mobilize a different secreted factor from adipocytes that controls vitellogenesis? Treatment of *ex vivo* cultured larval brains with adiponectin results in ILP secretion via AdipoR (Kwak et al. 2013). While the majority of *Drosophila* ILPs are produced in the medial neurosecretory cells (MNCs) in the brain, DILP6 is produced in the fat body (Okamoto et al. 2009, Bai et al. 2012); is it secreted in response to an adiponectin-like signal? The effect of MNC ablation on oogenesis is substantial, but mild when compared to the effect of removing *InR* from the germline(LaFever and Drummond-Barbosa 2005), suggesting that another source of ILPs (such as DILP6) or a requirement for basal InR activity in vitellogenesis. Overall, studies of the role of AdipoR in tissues outside of the

ovary would provide a deeper understanding of the adiponectin signaling axis in flies, possibly identifying new genetic interactors and routes of inter-organ communication impinging on the ovary.

Multiple nutrient-dependent signals from the fat body regulate the GSC lineage

In addition to my unpublished work describing potential roles for AdipoR in the adult fat body, my research has helped to characterize other adipocyte-specific signaling pathways required for normal oogenesis. In Chapter V, I present a published study I performed in conjunction with Alissa Armstrong. There, we demonstrate that knockdown of single amino acid transporters in adult adipocytes results in increased GSC loss over time and that this effect is independent of TOR (Figures 5.6-7). Adipocyte TOR signaling does, however, control ovulation, and amino acid signaling likely contributes to this effect (Figures 5.11-12), in concert with other mechanisms. That GSC loss occurs with knockdown of a single amino acid transporter in adipocytes demonstrates the exquisite sensitivity of GSC maintenance to the physiological environment; indeed, maintenance appears to be much more tightly regulated than ovulation. Perhaps regulation of ovulation is less sensitive because oocytes at that stage have already passed through several nutrient checkpoints, including the particularly energy-intensive process of vitellogenesis. Alternately, dramatic changes in ovulation may depend more heavily on rapid responses to the egg deposition environment; *Drosophila* are notably selective about where they lay their eggs and integrate multiple criteria to evaluate the suitability of any given location (Yang et al. 2008). Since octopaminergic neurons innervate the ovary and are required for ovulation (Lee et al. 2003, Monastirioti 2003, Deady and Sun 2015), it would be interesting to see if TOR signaling feeds into this system or represents an alternate mechanism to modulate ovulation.

What are the signals acting downstream of the amino acid response (AAR) pathway in adipocytes to regulate GSC maintenance? AAR pathway activation both decreases global translation and increases levels of activating transcription factor 4 (ATF4) and its targets (Murguia and Serrano 2012). Our observation that adipocyte morphology is grossly unaffected upon adipocyte amino acid transporter knockdown (Figure 5.4) argues against general adipocyte dysfunction as a cause of GSC loss, suggesting instead specific changes that have downstream consequences for the ovary. Future work in the Armstrong lab at the University of South Carolina will address the relative contributions to GSC loss of generation translation and ATF-dependent transcription downstream of the AAR pathway. It will be interesting to see what, if any, of these AAR pathway targets were uncovered in the iTRAQ proteomic analysis.

The identification of *3.1Lsp2-Gal4* as an adult adipocyte-specific adipocyte driver in female flies (Figure 5.2-3) provides a useful tool for future research into fat body function. Our experience testing the expression patterns of drivers described in the literature to be specific to the adult fat body or adipocytes emphasizes the importance of carefully characterizing those patterns when attempting physiological studies. Further studies should seek to identify other suitable ways to genetically manipulate the fat body, especially because this particular driver is not induced on a poor diet, limiting its experimental usefulness (Figure 5.1). Many aspects of adult fat body biology, including its developmental timing, remain unclear. Is the fat body regionalized as we know the larval fat body and the adult gut to be? As described in Marianes and Spradling (2013), where expression patterns of different drivers were used to describe regions of the adult gut, understanding different regions of the adult fat could provide insight into its many functions. Are there gross

morphological differences between abdominal fat, the focus of most of our work, and the fat cells found throughout the fly body? Understanding the fundamental biology of adult adipocytes will allow us to ask more complex questions about its participation in organismal physiology.

AMPK intrinsically and differentially regulates both the GSC and FSC lineage

Chapter IV describes our ongoing effort toward understanding the role of AMPK in *Drosophila* oogenesis. Consistent with a model in which low cellular energy activates AMPK, preliminary data suggest that *AMPK* mutant GSCs and follicle cells do not downregulate their proliferation in response to poor diet (Figures 4.2, 4.5), suggesting an active role for AMPK when nutrients are limiting. Several other results, however, indicate that AMPK is also required for other cellular functions under favorable energy conditions. For example, *AMPK* mutant GSCs are lost from the niche when flies are fed a rich diet, but not any more frequently than control GSCs when cultured on a poor diet (Figure 4.1). Furthermore, AMPK is required intrinsically for follicle cell growth regardless of diet (Figure 4.4), although studies from another group indicate that the effect is more dramatic on a poor diet(Haack et al. 2013) . AMPK also appears to be required for cell survival under normal dietary conditions, although the affected cells have not yet been determined (Figure 4.7). Finally, we observe a diet-independent role for AMPK in regulating follicle cell germline cyst encapsulation and are currently evaluating potential mechanisms involved (Figure 4.6).

Are the roles of AMPKα in regulating germline cyst encapsulation and GSC maintenance moonlighting roles, completely separable from its central role in regulating

energy homeostasis via a functional AMPK heterotrimer? Knocking out a single subunit of the AMPK heterotrimer leads to complete loss of AMPK activity (Woods et al. 1996, Dyck et al. 1996, Hardie 2003); however, whether individual subunits functionally associate with proteins outside of this complex has not been explored. Studies of the *Drosophila* AMPKβ and γ regulatory subunits describe nutrient-independent, neuroprotective functions. Retinal clones of *alicorn*, the *Drosophila* AMPKβ, gradually degenerate with exposure to light, although the mechanism is unknown (Spasic et al. 2008). Additionally, an AMPKγ allele with disrupted transcription of a neuronally expressed isoform is named *löchrig*, from the German meaning "full of holes", because of the extensive brain vacuolization and neural degeneration observed in the mutant (Tschape 2002). However, these phenotypes could be explained by the previously characterized role for AMPK in regulating autophagy. Indeed, AMPKγ global mutants do not induce autophagy in the larval fat body in response to ecdysone (Lippai et al. 2008). While autophagy is activated under cellular stress conditions, including nutrient deprivation, it also plays a role in development and normal cellular homeostasis (McPhee and Baehrecke 2009). Therefore, one possibility is that AMPK constitutively regulates processes that are often associated, but not exclusive to, nutrient stress, and that upon further activation by AMP or ADP, its increase in activity generates a more dramatic effect.

AMPK may interact with signaling pathways already implicated in progenitor or ovarian activity. For example, AMPK is a downstream effector of adiponectin signaling in many cellular contexts (Ye and Scherer 2013); we rule out this mode of action in the context of ovarian GSCs, however, because *AMPK* mutant GSCs are not lost more frequently than controls on a poor diet, as is the case with *AdipoR* mutant GSCs. In Chapter IV, I discuss the

possibility that AMPK acts via TOR, Hedgehog, or Notch signaling pathways to control various aspects of ovarian function. Additionally, Yorkie (Yki), canonically a downstream effector of the Hippo cell growth and proliferation pathway (Zhao et al. 2011), is suppressed by AMPK in a Hippo-independent pathway found in asymmetrically dividing larval central brain neuroblasts (Gailite et al. 2015). Hippo-independent Yki activation has also been observed in the somatic cells of the fly ovary (Sarikaya and Extavour 2015). Furthermore, recent studies have shown that a physical interaction between Yki and taiman (tai), a transcriptional coactivator of ecdysone signaling, regulates the expression of a set of target genes distinct from the those induced by Hippo pathway activation (Zhang et al. 2015). Is Yki hyperactivation responsible for the apparent overproliferation of *AMPK* mutant GSCs and follicle cells on a poor diet? While *tai* mutant GSCs proliferate normally under well-fed conditions (Ables and Drummond-Barbosa 2010), proliferation under nutrient stress has not been assessed. *Yki* mutant GSCs are maintained at the niche (Shcherbata et al. 2007), suggesting that, should this pathway be involved in AMPK-mediated proliferation, an additional AMPK target would be responsible for the GSC maintenance defect. None of this evidence is disqualifying, however, and future studies should address the possible involvement of Yki in ovarian AMPK signaling.

A second model for regulation of AMPK activation involves the activity of its upstream kinases or phosphatases under particular cellular conditions. Liver kinase B 1 (LKB1), the best-described AMPK kinase, associates with stabilizing proteins STE-related adaptor (STRAD) and mouse protein 25 (MO25) in the cytoplasm (Baas et al. 2003, Boudeau et al. 2003), where it appears to constitutively phosphorylate the AMPKα catalytic subunit (Hawley et al. 2003). This suggests that a major point of regulation of AMPK is not LKB1,

but the presence of AMP or ADP, which, upon binding to the γ subunit, induce a conformational change that compromises dephosphorylation of AMPKα (Hawley et al. 2003). Intriguingly, pharmacological agents that bind a pocket between the α and β subunits of AMPK, activating the enzyme independently of AMP, have been identified; whether any metabolites are capable of activating AMPK through this mechanism, however, remains unknown (Hardie 2015).

For my remaining time in the lab, I will focus on finalizing experiments characterizing the ovarian role of AMPK and addressing potential AMPK targets in different cell types. Additionally, with an undergraduate in the lab, Ondina Palmeria, I am generating genetic mosaic *alicorn* mutants. It will be interesting to test if other subunits of AMPK have AMPKα-independent roles in the ovary.

Final thoughts and public health implications

Understanding the many layers of regulation that impinge on stem cell populations is an interesting basic biological problem with medical and public health implications. Stem cell populations support many adult tissues, and the activities of these cells can be disrupted in aging and disease states (Ables et al. 2012). Dysregulation of adipocytes, as found in lipodystrophy and obesity, is often associated with multiple pathologies, including cancer (Ye and Scherer 2013). In addition to the well-characterized overlap between gene expression in cancer and stem cells (Baylin 2008), nutrient-sensing pathways are co-opted by many tumors (Chen 2011, Jee et al. 2005), making our understanding of physiological nutrient sensing inextricable from our understanding of disease states.

Physiological regulation of insect oogenesis as a whole has tremendous public health implications. The ovaries of anautogenous mosquitos—including *Aedes aegypti*, the yellow

fever and Zika virus vector (Marchette et al. 1969)—remain arrested in a previtellogenic state until consumption of a blood meal (reviewed in Smykal and Raikhel 2015)], thus providing a dramatic example of the nutritional regulation of oogenesis. How similar is the physiological regulation of oogenesis in disease vectors to that of *Drosophila melanogaster*? Many of the nutrient-dependent pathways that control fly oogenesis, including insulin, TOR, and ecdysone signaling, play a role in regulating mosquito oogenesis [Chapter I; (Smykal and Raikhel 2015)], although a dearth of genetic tools in mosquitoes has complicated cell-specific manipulations of these pathways. The full physiological response of *A. aegypti* to the blood meal is an active area of investigation, and GSC activity, while likely present, has never been formally characterized in this species. On the other hand, a major hurdle to current mosquito control efforts is effectively spreading genetically modified insects into the general, potentially disease-carrying, population (Luckhart et al. 2010). A good understanding of how to manipulate the reproduction of such modified insects, including through these nutrient sensing pathways, could allow them to outcompete mosquitoes in the wild. We hope that our work and the work of others will inform studies of mosquito oogenesis that will help limit the spread of vector-borne diseases.

APPENDIX:

CONTROLLING *DROSOPHILA* DIET WITH MUTANT LABORATORY YEAST

Introduction

Work in this dissertation (and in the Drummond-Barbosa lab) primarily addresses *Drosophila* oogenesis under two nutrient conditions: rich and poor diet. "Rich diet" consists of standard fly medium (a combination of yeast extract, cornmeal, molasses, and agar) supplemented with wet yeast paste, and "poor diet" solely of molasses and agar (Drummond-Barbosa and Spradling 2001). Many nutrient-sensing pathways regulating female gametogenesis in *Drosophila* have been identified, including insulin and Tor signaling (see Chapter I for details). However, little is known about the specific dietary factors that modulate the ovarian response to diet. Since the *Drosophila* diet consists primarily of yeast, itself a genetically tractable model organism, we conceived of a screen to identify factors modulating oogenesis in adults. While I did not carry out this screen, this chapter provides a proof of principle for the use of *Saccharomyces cerevisiae* from the yeast knockout collection (Winzeler et al. 1999) to identify dietary factors that control *Drosophila* oogenesis.

Methods

Yeast cultures and knockout verification

Yeast strains were cultured from glycerol stocks on Yeast Peptone Dextrose (YPD; per 1 L final volume: 24 g Bacto agar, 20 g Bacto peptone, 10 g yeast extract, with 50 mL sterile 40% w/v glucose added after autoclaving) plates at 30°C. The genetic

identities of single colonies were confirmed by PCR based on the instructions provided by the yeast knockout collection [(Winzeler et al. 1999), Figure A.1]. Briefly, single colonies were incubated in 3 mL YPD culture with shaking at 30 °C overnight. 20 μL of cells were lysed with 0.5 mm glass beads in 100 μL 0.6M sorbitol/10 mM HEPES (pH 7.4) by vortexing for 5 minutes. 1 μL of the lysed sample was used for PCR with EconoTaq (Lucigen) according to the manufacturer's instructions. Patches from single colony streaks were spread in thin layers on room temperature molasses plates for use in egg counts.

Egg counts

To measure egg production, five pairs of 0-3 day old females and males of the indicated genotypes were cultured in plastic bottles containing molasses/agar plates alone or supplemented with a thin layer of yeast paste (from stock of 0.6g Red Star Active Yeast/mL water), yeast extract (from stock of 5 g/mL water), or yeast from the knockout collection (see above). Egg counts were performed in triplicate. Flies were maintained between 23-25 °C, and the plates were replaced daily for 5-8 days. Photographs of eggs were acquired with a Zeiss Axioimager A.2.

Results

A lab strain of *S. cerevisiae* maintains Drosophila fecundity

In order to use the yeast knockout collection to identify important factors for oogenesis, I first established that a haploid laboratory strain of yeast, *BY4741 Matα*, could substitute for yeast paste in promoting *Drosophila* fecundity. This is the genetic background of the yeast knockout collection, a comprehensive collection of viable yeast

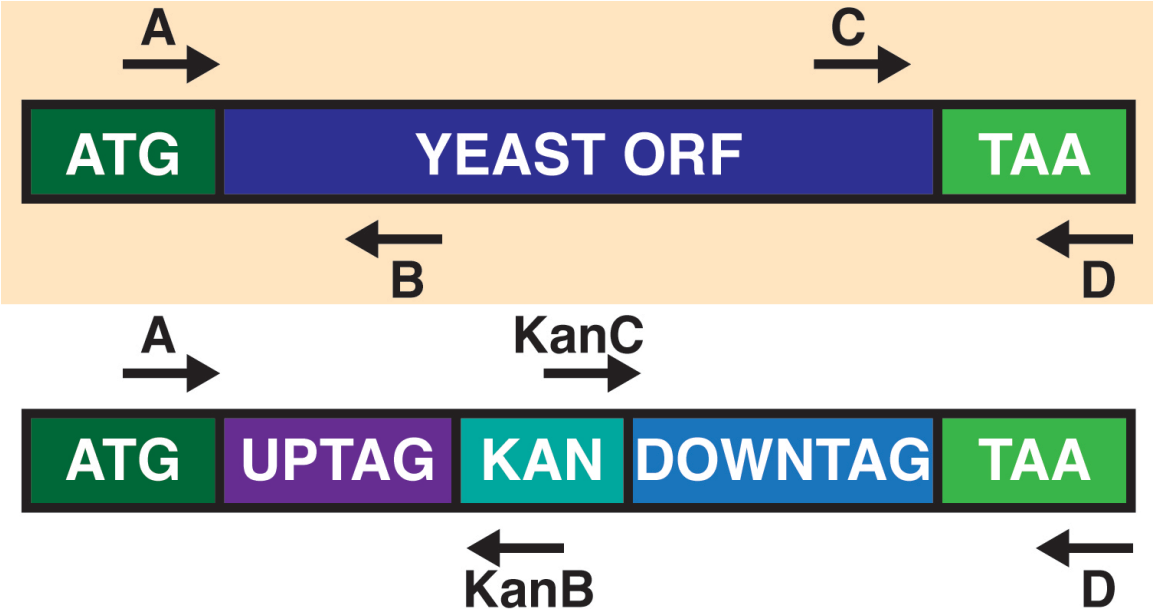


Figure A.1. PCR strategy for confirmation of yeast mutants in the knockout collection. A specific yeast ORF is detected by specific primer pairs specific (top). If that ORF has been replaced with a kanamycin cassette, the KanB and KanC instead pair with ORF-specific pairs (A and D, respectively) to positively identify mutants by PCR (below). For primer sequences used in this study, see Table A.1. Adapted from (Winzeler et al. 1999).

Table A.1. Primers used for yeast knockout collection verification

KanB	*erg4*
DDB443	**A-DDB433**
5’-CTGCAGCGAGGAGCCGTAAT-3’	5’-GATCATGTGTAATAGGCGAATAAGG -3’
KanC	**B-DDB434**
DDB444	5’-CAAAACGGTCAATAATGACATACAA-3’
5’-TGATTTTGATGACGAGCGTAAT-3’	**C-DDB435**
erg2	5’-AGTTTGGATTTATGCTGATCTTCTG-3’
A-DDB557	**D-DDB436**
5’-TATGGAAGAATTTGGATAGATCTGC-3’	5’-CCATATTAATGAAGAATCATGGGAG-3’
D-DDB559	***erg5***
5’-CAGTCTGCTATGTTGATTCTGCTTA-3’	**A-DDB563**
A-2-DDB558	5’-TTGTCTTCAGGCAAAGGGACTG-3’
5’-ATATATCCGTCGTCGTAGGTGATAA-3’	**D-DDB564**
D-2-DDB560	5’-GTCGCACCTTTAGCAGATCATTAGC-3’
5’-CGAAGTTTACACTCCTGGTATGACT-3’	***erg6***
erg3	**A-DDB437**
A-DDB561	5’-CTGTTGCCGATAACTTCTTCATTGC-3’
5’-ATAAGTGGCACAATAGAAGGTGAAG-3’	**B-DDB438**
D-DDB562	5’-TATCGGTTCTACCATCCCAATTTCTCA-3’
5’-TTTTAAAGCTTCCAGCTTCCTATTT-3’	**C-DDB439**
	5’-TACGTTCAAAACTTAGCTAATTTGGCC-3’
	D-DDB440
	5’-GGCCTGCTAGCAATGAACGTGCTA-3’

mutants (Winzeler et al. 1999). Commercially available yeast, commonly used in *Drosophila* lab diets, is diploid and varies from laboratory haploid strains at multiple single nucleotide polymorphism loci (Ben-Ari et al. 2005). During five days of feeding, female flies cultured on *BY4741 S. cerevisiae* laid a comparable or slightly lower number of eggs to those fed yeast paste (Figure A.2A). Importantly, this pattern is maintained in several wild type fly strains (Figure A.2B,C). This confirms that *BY4741* yeast can support late processes in oogenesis, including ovulation and egg deposition. The timing of these experiments, however, precludes investigation of the effect of the laboratory yeast on GSC activity, as the time between GSC division and egg deposition is approximately 10 days and the experiments were stopped after five.

Ergosterol biosynthesis in dietary yeast is required for normal *Drosophila* fecundity and egg quality

As previously reported, flies maintained on yeast extract, the water soluble portion of autolyzed yeast, deposit fewer eggs than those fed yeast paste (Bass et al. 2007), comparable to those fed a poor diet (Drummond-Barbosa and Spradling 2001) (Figure A2). The inability of yeast extract to sustain *Drosophila* fecundity could reflect multiple dietary requirements. One possibility is that water-insoluble compounds, including lipids, are integral for Drosophila fecundity. For example, *Drosophila*, like other insects, are sterol auxotrophs (Hobson 1935), and dietary sterol is required for membrane integrity and production of the steroid hormone ecdysone, a major regulator of oogenesis (Carvalho et al. 2010). To test the effectiveness of yeast manipulation in *Drosophila* dietary control, we fed flies viable *S. cerevisiae* mutants in the ergosterol

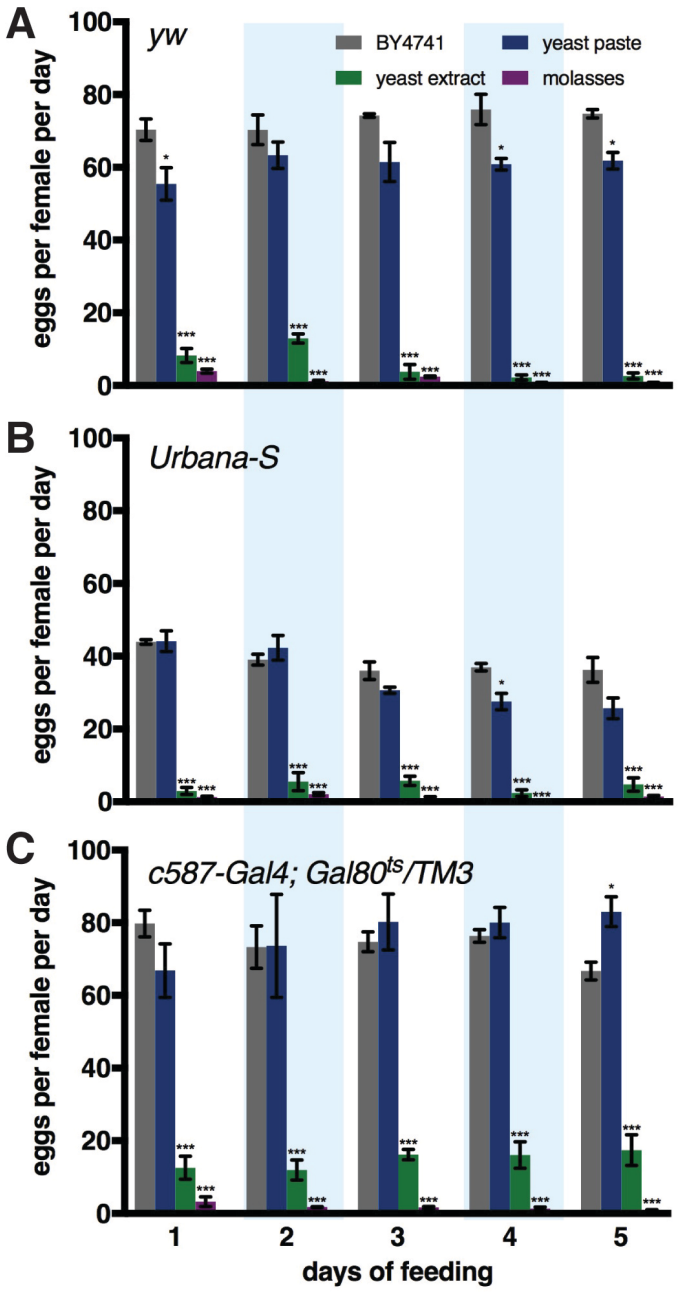


Figure A.2. ***BY4741*** **yeast supports normal fecundity in different strains of** ***Drosophila*****.** Egg counts from *yw* (A) and *Urbana-S* (B) genetic backgrounds and *c587-Gal4*; *Gal80^{ts}/TM3* (C) flies cultured on *S. cerevisiae*, standard laboratory yeast paste, yeast extract, and molasses plates alone. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Student's *t* test compared to *BY4741*.

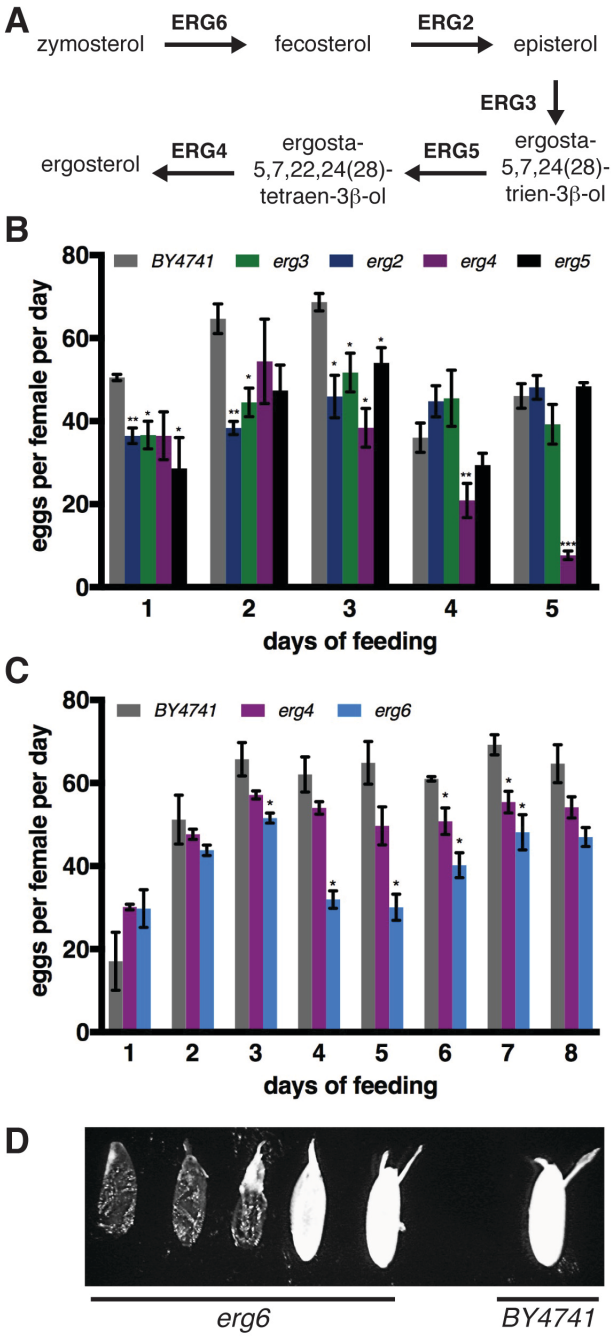


Figure A.3. Ergosterol biosynthesis in dietary yeast influences *Drosophila* fecundity and egg development. (A) Ergosterol precursors are modified by a series of enzymes to generate ergosterol. Adapted from (Tiedje et al. 2007). **(B, C)** Ergosterol mutant strains of *S. cerevisiae* do not support fecundity at the level of *BY4741* (wild type) yeast. **(D)** The collapsed, transparent eggs observed when flies are cultured with *erg6* compared to the normal morphology of those fed wild type *BY4741* yeast. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Student's *t* test compared to *BY4741*.

synthesis pathway (Figure A.3) and evaluated their fecundity. Overall, flies maintained on ergosterol mutant strains of yeast trend toward lower fecundity than those fed wild type yeast (*BY4741*), although changes at these early time points are subtle (Figure A.3B,C). There does not appear to be a relationship between the position of the enzyme in the biosynthetic pathway and the severity of defect, suggesting that particular ergosterol precursors may be suitable for some sterol functions in *Drosophila*. Indeed, recent work demonstrates that *erg4* mutant yeast can support fly development through adulthood (Lavrynenko et al. 2015), indicating that this is at least the case for ergosta-5,7,22,24(28)-tetraen-3β-ol. In addition to their reduced fecundity, *erg6*-fed *Drosophila* lay eggs with dramatic morphological defects, where the egg appears transparent and/or collapsed, suggesting a defect in yolk deposition or in egg shell formation (Figure A.3D). These eggshells are similar to those observed in *endos* mutants; *endos* has a role in meiotic maturation (Drummond-Barbosa and Spradling 2004). This phenotype was not observed in other ergosterol mutants, suggesting that the metabolite modified by ERG6, zymosterol, is the final ergosterol precursor that cannot be used in these processes in *Drosophila*. We cannot exclude the possibility, however, that an accumulation of another metabolite, including zymosterol itself, is detrimental to yolk deposition or egg shell formation. Interestingly, diet-derived zymosterol makes up a large proportion of sterols in the larval gut; whether this accumulation has a functional significance or simply reflects an inability to use zymosterol in other processes remains unknown (Carvalho et al. 2012).

Discussion

The inability of yeast extract to support *Drosophila* fecundity leads us to ask what factors the extract lacks that are present in yeast paste. In addition to an alteration of odors and other short-lived metabolites found in living organisms, yeast extract lacks insoluble

components of live active yeast, including lipids, and perhaps other biologically active peptides that are destroyed in the autolytic process. While several groups have lead efforts to standardize *Drosophila* culture media through chemically defined food for *Drosophila* laboratory use, it remains unclear whether those diets reflect the full complement of factors required to maintain normal physiology or robust fecundity (Piper et al. 2014, Lee and Micchelli 2013). Since laboratory yeast supports fecundity at a level near that of active yeast paste in multiple genetic backgrounds, we suggest that the yeast knockout collection could provide a strong foundation for understanding the metabolites, especially lipids, involved in regulating *Drosophila* oogenesis. For example, viable lipid biosynthesis mutants in the yeast knockout collection could lead to valuable insights into specific dietary lipid requirements in *Drosophila* oogenesis.

The use of live, mutant organisms as food could present more difficulty in interpreting results than medium drop-out experiments. Disruption of a metabolic pathway can have pleiotropic effects, and multiple pathway mutants should be tested to implicate a signaling cascade. In particularly promising candidates, the yeast metabolome could theoretically be generated, potentially uncovering multiple candidate metabolites. Dietary supplementation rescue experiments can be conducted to pinpoint particular dietary factors. Finally, once factors are identified in yeast, candidate genes involved in those metabolic or signaling pathways in *Drosophila* could be tested for a role in oogenesis.

Egg counting assays can vary widely from experiment to experiment, and their outcomes reflect a host of behavioral and biological processes. As oogenesis is exquisitely controlled, so is egg deposition, and several genetic manipulations in *Drosophila* can block ovulation (Armstrong et al. 2014, Deady et al. 2015). The early time points used in these

particular assays precludes our comment on the effect of ergosterol mutant feeding on early germline processes, since cystoblasts take approximately 10 days to produce stage 14 oocytes (Spradling 1993). Regardless, these pilot studies lay out a possible strategy for the identification of novel diet-dependent factors regulating *Drosophila* oogenesis.

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CURRICULUM VITAE

KAITLIN M. LAWS
Johns Hopkins Bloomberg School of Public Health
Department of Biochemistry and Molecular Biology
615 N. Wolfe Street W3104-2 Baltimore, MD 21205
240-449-0151 klaws@jhu.edu

EDUCATION

2009-2016 **Johns Hopkins Bloomberg School of Public Health**, Baltimore, MD
Ph.D. in Biochemistry and Molecular Biology
Dissertation: "Physiological control of the *Drosophila melanogaster* ovary"
Advisor: Daniela Drummond-Barbosa, Ph.D.

2009 and **Clemson University**, Clemson, SC
B.S. in Biochemistry, Calhoun Honors College, Magna Cum Laude
Honors thesis: "Biochemical and kinetic characterization of acetate kinase phosphotransacetylase of *Phytophthora ramorum*"
Advisor: Kerry Smith, Ph.D.

OTHER RESEARCH EXPERIENCE

2008 **Summer undergraduate researcher**, Jackson Laboratory, Bar Harbor, ME
Project: "Characterizing the *repro8* male infertility phenotype in male mice"
Advisor: Mary Ann Handel, Ph.D.

HONORS and AWARDS

2014 **John Scocca Award for Excellence in Research**
Department of Biochemistry and Molecular Biology

2012 **Elsa Orent Keiles Fellowship**
Department of Biochemistry and Molecular Biology

2009 **Outstanding Senior in Biochemistry and Genetics**
Clemson University

2009 **SC Life/HHMI Scholarship**

2008 **SC Life/HHMI Scholarship**

TEACHING

2016 **Teaching assistant, Stem Cells and the Biology of Aging and Disease**
Department of Biochemistry and Molecular Biology

2015 **Guest lecturer, Cell Stress and Aging**
Department of Biochemistry and Molecular Biology

MENTORING

Lauren McGinnis, JHSPH BMB Ph.D. student
Paige Marsh, JHU undergraduate
Soo-ah Park, Towson University undergraduate
Edward Culbertson, JHSPH BMB Ph.D. student
Robert MacPherson, JHSPH BMB Ph.D student
Aline Dantes-Rodriguez, JHU summer undergraduate researcher
Ondina Palmeira, JHU undergraduate

PRESENTATIONS

Laws, K.M. and Drummond-Barbosa, D. (2016). The control of *Drosophila* GSCs by diet and physiology. Invited by Michael Strand, Department of Entomology, University of Georgia, Athens, GA.

Laws, K.M. and Drummond-Barbosa, D. (2016). The control of *Drosophila* GSCs by diet and physiology. Invited by Craig Montell, Department of Neuroscience, University of California, Santa Barbara, Santa Barbara, CA.

Laws, K.M. and Drummond-Barbosa, D. (2016). The control of *Drosophila* GSCs by diet and physiology. Invited by Greg Bashaw, Department of Neuroscience, University of Pennsylvania, Philadelphia, PA.

Laws, K.M. and Drummond-Barbosa, D. (2015). The control of *Drosophila* GSCs by diet and physiology. Department of Biochemistry and Molecular Biology Retreat, Baltimore, MD.

Laws, K.M., Sampson, L.L., and Drummond-Barbosa, D. (2014). An insulin-independent requirement for the adiponectin receptor homolog in the maintenance of *Drosophila melanogaster* germline stem cells. 55th Annual *Drosophila* Genetics Conference, San Diego, CA.

Laws, K.M., Sampson, L.L., and Drummond-Barbosa, D. (2012). Investigating the role of the adiponectin receptor in *Drosophila melanogaster* oogenesis. Department of Biochemistry and Molecular Biology Colloquium, Baltimore, MD.

Laws, K.M., Sampson, L.L., and Drummond-Barbosa, D. (2011). Investigating the role of the adiponectin receptor and of phosphosphingolipids in during *Drosophila* oogenesis. Department of Biochemistry and Molecular Biology Retreat, York, PA.

SELECTED POSTERS

Laws, K.M., Armstrong, A.A., Weaver, L.N., Ma, T., Matsuoka, S., Munoz, I., Drummond-Barbosa, D. (2016). Control of germline stem cell lineages by a dynamic physiological environment. Department of Biochemistry and Molecular Biology Centennial Celebration, Baltimore, MD.

Laws, K.M., Sampson, L.L., and Drummond-Barbosa, D. (2014). An insulin-independent role for the adiponectin receptor in *Drosophila melanogaster* oogenesis. Germ Cells Meeting, Cold Spring Harbor, NY.

Laws, K.M., Sampson, L.L., and Drummond-Barbosa, D. (2013). The role of the adiponectin receptor homolog in *Drosophila melanogaster* oogenesis. 54th Annual *Drosophila* Genetics Conference, Washington, DC.

Laws, K.M., Sampson, L.L., and Drummond-Barbosa, D. (2012). The role of the adiponectin receptor homolog in *Drosophila melanogaster* oogenesis. 53rd Annual *Drosophila* Genetics Conference, Chicago, IL.

PUBLICATIONS

Laws, K.M. and Drummond-Barbosa, D. (in preparation). The role of AMPK in Drosophila melanogaster oogenesis.

Laws, K.M. and Drummond-Barbosa, D. (in press). Physiological control of germline stem cell lineages. Results and Problems in Cell Differentiation.

Laws, K.M. and Drummond-Barbosa, D. (2015). Genetic mosaic analysis of stem cell lineages in the Drosophila ovary. Methods Molecular Biology, 1328:57-72.

Laws, K.M., Sampson, L.L., Drummond-Barbosa, D. (2015). Insulin-independent role of adiponectin receptor signaling in Drosophila germline stem cell maintenance. Developmental Biology, Mar 15;399(2):226-36.

Armstrong, A.R., **Laws, K.M.,** Drummond-Barbosa, D. (2014). Adipoctye amnio acid sensing controls adult germline stem cell number via the amino acid response pathway and independent of Target of Rapamycin signaling in Drosophila. Development, Dec;141(23):4479-88.

Baron, J.A., **Laws, K.M.,** Chen, J.S., Culotta, V.C. (2013). Superoxide triggers an acid burst in Saccharomyces cerevisiae to condition the environment of glucose-starved cells. Journal of Biological Chemistry, Feb 15;288(7):4557-66.

Ables, E T, **Laws, K.M.,** Drummond-Barbosa, D. (2012). Control of adult stem cells in vivo by a dynamic physiological environment: diet-dependent systemic factors in Drosophila and beyond. Wiley Interdisciplinary Reviews Developmental Biology, Sep-Oct;1(5):657-74.

PROFESSIONAL ACTIVITY AND SOCIETY MEMBERSHIPS

2014-2015 Coordinator, student-invited seminar series
Department of Biochemistry and Molecular Biology

2009-2010 Sigma Xi Honors Research Society